REGULATION OF

Isopentenoid Metabolism

EDITED BY

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Regulation of Isopentenoid Metabolism

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Foreword

THE ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the Series parallels that of the continuing ADVANCES IN CHEMISTRY SERIES except that, in order to save time, the papers are not typeset, but are reproduced as they are submitted by the authors in camera-ready form. Papers are reviewed under the supervision of the editors with the assistance of the Advisory Board and are selected to maintain the integrity of the symposia. Both reviews and reports of research are acceptable, because symposia may embrace both types of presentation. However, verbatim reproductions of previously published papers are not accepted.

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Preface

ISOPENTENOIDS ARE A CLASS OF LIPIDS that are vital to all aspects of growth, development, and reproduction in living systems. In proliferating cells, the isopentenoid pathway gives rise to primary metabolites such as sterols, which are necessary components for membrane structure and function, and to secondary metabolites (usually in growth-arrested cells or unique compartments), which are formed after sterol production for other purposes. These secondary metabolites may or may not be important to the specific cell type that produces them but are nevertheless essential to survival (monoterpenes) and to reproductive fitness (hormones). Developmental change can be negative (as in cancerous cells where regulatory strictures are lost or in the deadly accumulation of cholesterol in human arteries) or positive. In either case, an important key to this change is the regulation (or lack thereof) of isopentenoid genesis.

Because the natural product chemistry of isopentenoids has been determined and there have been several major recent advances in analysis, the biochemistry of these compounds can now be investigated. To present an overview of this rapidly escalating field, several divisions of the American Chemical Society and the Federation of American Societies for Experimental Biology (FASEB) (HMGCoA-reductase satellite session) jointly sponsored a four-day symposium comprising more than 25 plenary lectures and a poster session. Perhaps the most important outcome of the symposium was a cross-fertilization of research efforts by the medical and agriculture communities to solve common problems regarding regulation of the isopentenoid pathway. The pathway operates similarly in animals and plants, but with different control points (rate-limiting enzymes) in different organisms and tissues. The design of inhibitors specific for the various regulatory steps is currently at the cutting edge of this effort because of their importance in improvement of animal and plant health. Consequently, the contributors selected for this book, for the most part, address the rational design of potent inhibitors of isopentenoid production that occurs before and after the cyclization of squalene

In Regulation of Isopentenoid Metabolism; Nes, W., et al.;
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oxide to sterols. Additional chapters cover newly emerging areas of isopentenoid research, including prenylated proteins and cancer, carotenoids and biotechnology, sterol homeostasis, and molecular biology.

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NOTE: Modern usage for the classical terms isoprenoids and terpenoids, see Nes and McKean, *Biochemistry of Steroids and Other Isopentenoids;* University Park Press: Baltimore, 1977.

Chapter 1

Biosynthesis and Utilization of Isoprenoids

Overview of Research Directions

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Mevalonic acid biosynthesis and its subsequent metabolism into a plethora of isoprenoid compounds has reemerged as a prominent field of study in chemical and biochemical science. The conversion of mevalonic acid into key intermediates of cellular metabolism, as structural components of cellular membranes, as natural products of significant commercial utility, and as natural protectants against infection and disease highlights the significance of this biochemical pathway. Additionally, the isoprenoid pathway has served as the target for important therapeutics and agrochemicals used to combat such diverse diseases such as fungal infections and infestations, hypercholesterolemia, and atherosclerosis. The contents of this volume will expand on these topics with details on the chemical, biochemical, and molecular biological progress made in this field presented at two recent ACS symposia.

I have been given the assignment to provide an overview of the scientific directions in the isoprenoid field. For this purpose, I have reflected upon recent symposia proceedings and have written my thoughts in a manner which I believe demonstrate the breadth of science involved in furthering our understanding and specific knowledge of the isoprenoid pathway.

Contributing authors to this volume were solicited from speakers at the ACS National Meeting held April 14-19, 1991 and the ACS/FASEB Symposium on the Biosynthesis and Utilization of Isoprenoids (Reductase V) held April 18-20, 1991 in Atlanta, GA. These two symposia attracted over 400 participants to a combined total of 15 scientific sessions held over the course of one week. Reflected in this volume are many diverse aspects of isoprenoid

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metabolism which have at their root a common biosynthetic pathway. The last compilation of such a wide range of topics associated with isoprenoids appeared in 1981 in a two volume set entitled "Biosynthesis of Isoprenoid Compounds," edited by John W. Porter and Sandra L. Spurgeon. That work detailed the major metabolic processes associated with isoprenoid biosynthesis which should serve the reader as a foundation and reference for entry into this field.

The Biochemical Pathway to Polyisoprenoids

Enzymology of the Pathway.

Formation of Mevalonic Acid. Our seeming ease of acceptance that mevalonic acid serves as the immediate precursor to the diverse structural class we call isoprenoids overshadows the extensive effort and work which lead to this remarkable discovery (*1*). Indeed emphasis in recent years has focused on the detailed enzymology of mevalonic acid formation from 3β-hydroxy-3-methyglutaryl coenzyme A (HMG-CoA) catalyzed by the enzyme HMG-CoA reductase (*2*). In fact, the basis for past symposia in this series (*3*), and the reason for the Reductase V subtitle in the title for the ACS/FASEB symposium, has been to review progress made in understanding the reductase biochemically and to exploit these findings through chemical applications. It is ironic, therefore, that only seven presentations at these symposia involved discussions of reductase specifically. These include elucidation of mevalonic acid formation via two related HMG-CoA reductase isozymes in plants. The relationship of these two enzymes to themselves and the overall isoprenoid pathway should be forthcoming with cloning of the two proteins and continued work on their biochemistry. In mammalian systems, the importance of HMG-CoA reductase as a therapeutic target continues to deliver increasingly more potent inhibitors. Alternative approaches to blocking mevalonate production via regulation of reductase gene expression with oxygenated sterols also remains an active research area. We should expect to see new developments in this field for years to come.

Isomerase, Polyprenyl Sythetase, and Prenyl Transferases. The molecular mechanisms underlying formation and utilization of the isoprenoid unit is benefitting from molecular biological approaches. Cloning of the enzymes with subsequent site directed mutagenesis is leading to a greater understanding of the reaction mechanisms for the isomerase and prenyl transferase enzymes. Advances in our understanding of gene regulation are also being made in mammalian systems through investigation of the prenyl transferase enzymes. Important new discoveries can be anticipated as the sequence homology as well as diversity of polyprenyl synthetases from different species become evident. The functional significance of various protein domains will be unraveled in the coming years. Applications of this information in the design and synthesis of new chemical entities with utility in the agrochemical and pharmaceutical industries should be anticipated and will certainly advance this field.

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Points of Divergence Along the Pathway. Formation of polyisoprenoids provides the basis for the diversity associated with the isoprenoid pathway. Historically, Ruzicka masterfully solved the structural diversity of many of the cyclic mono (C_{10}) -, sesqui (C_{15})-, and di (C_{20}) terpenoids through the application of Wallach's "isoprenoid rule" (*1*). Today this work continues as questions of diversity at the enzymatic level are beginning to be answered. In the area of terpenoid biosynthesis, key questions remain as to how the seemingly related cyclase enzymes impart unique cyclization processes to a common acyclic substrate? How do the cyclases impose conformational restraints? How are reaction intermediates stabilized? What prevents destruction of the enzyme through autoalkylation reactions? Answers to these questions are being pursued and will be forthcoming with purification, cloning and mutagenesis studies of the enzymes involved.

Progress toward our understanding of diverse reaction mechanisms is also being made in the sterol branch of the pathway. Our understanding of squalene formation and the importance of squalene synthetase as a regulatory enzyme in sterol biosynthesis should advance more quickly with the molecular cloning of the yeast enzyme. Similarly, advances on squalene cyclization mechanisms using purified preparations will aid in inhibitor design. This is an extremely competitive area of research because of the importance which the synthetase plays in cholesterol biosynthesis. Inhibitors of this enzyme should serve as useful therapeutics to control elevated serum cholesterol levels (see below).

Further along the pathway, it has been shown that novel catalytic processes are associated with sterol formation. The 14α-formyloxy-lanost-8-en-3β-ol intermediate in lanosterol demethylation has been isolated and defines a new reaction mechanism catalyzed by a cytochrome P-450 species. Application of mechanistic data of this sort has lead to the design of extremely strong enzyme inhibitors directed at the demethylase and other sterol transforming enzymes more distal in the pathway. This is particularly true in the plant and fungal areas where inhibitory molecules can be expected to find application in various agricultural settings for either fungal or weed control.

Isoprenoid Natural Products

Rubber, Terpenoids, and Carotinoids. The importance of the isoprenoid pathway is not only evident through advances in our understanding of basic science and enzymology, but also in the key metabolic role and commercial potential of the end-products of the divergent pathways. Rubber is perhaps the most obvious example of a commercial application of end products of polyisoprene biosynthesis. Optimization of rubber production through genetic engineering and biotechnology will certainly lead to better sources and expanded growth opportunities for this material. Similarly, application of biotechnology to the growth and production of high carotenoid-containing algae is leading to natural sources for these materials which historically have been produced through organic synthesis. A greater demand for carotenoids in the pharmaceutical industry and as food colorants are the major factors behind these new process developments. An expanded use of terpenoids can also be invisioned with novel application in disease control.

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Sterols. Although I have already mentioned that sterol transforming enzymes serve as targets for inhibitor design, I think it is equally important to point out the utility of sterols as commercial products. Through a combination of classical fermentation technology coupled with molecular biology, genetics and bioengineering, it is now possible to accumulate virtually any isoprenoid generated along the pathway. As an example, yeast have been selected which over-produce sterols to levels up to 20% of the dry cell weight without significant growth impairment or harm to the organism. Thus, the use of yeast as a feedstock for isoprenoid precusors used in organic synthesis of vitamins, steroid hormones, or novel application can be anticipated in the years ahead. Extension of this technology, which results in accumulation of isoprenoid products, to other branches of the pathway could lead to organisms with altered isoprenoid end-product profiles with multiple application. For instance, it may be possible to design insect resistant plants through changes in the balance of end-products derived from the isoprenoid pathway. It can be invisioned that toxicity may be imparted to a parasite without alteration in host productivity by the appropriate selection of a host end-product isoprenoid profile, a steroid perhaps obtained through bioengineered metabolic blockade. Thus, a potentially environmentally safe method for insect control may be feasible through knowledge and manipulation of the isoprenoid pathway. Advances of this type will certainly be made within the next few years.

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Commercial Applications

Agrochemicals.

Fungal Growth Control. Historically the sterol branch of the isoprenoid pathway has served as a valuable target for fungicide development. Enzyme inhibitors directed at squalene epoxidation, sterol 14α-demethylation, and sterol nuclear bond rearrangements (sterol $\Delta^8 \rightarrow \Delta^7$-isomerase) have identified the allylamines, the azoles, and the morpholines as useful structural classes for controlling pathogenic fungi (*4*). These bountiful and successful discoveries have created an intense search for new and novel inhibitors of the sterol biosynthetic pathway. Application of highly specific, directed research at new enzyme targets such as 2,3-oxidosqualene cyclizase, sterol 4-demethylase, and the sterol C-24 alkylation sequence is ongoing along with searches for better inhibitors of "classical" enzyme targets. Application of highly sophisticated inhibitor design strategies using knowledge of enzyme reaction mechanisms is leading to extremely potent and potentially useful agents. These high energy intermediates (HEI) block catalysis in a mechanism specific manner and act as enzyme traps. One can expect this work to lead to highly useful agents for the agrochemical sector in keeping with the past tradition of sterol biosynthesis inhibitor as fungicides.

Herbicides. Our understanding of the enzymology of sterol biosynthesis is becoming increasing more refined. We are beginning to see that the subtle differences between species may allow for designing unique chemical inhibitors with exacting specificity. However, the commonalty of the biochemical process, and the necessity of isoprenoid products to support metabolic functions, is defining a new use for sterol biosynthesis inhibitors as herbicides and plant growth regulators. Again, the old and seemingly well characterized fungicide inhibitors are leading the way. We are finding that these agents are losing their defined specificity with age. The apparent unwanted side-effect of activity against host pathways is actually defining targets for future herbicide development. Indeed, unique agents can be envisioned which maintain the desired potency toward the isoprenoid biosynthetic pathway, but which undergo novel transformations prior to inhibition, or display novel uptake properties which can establish specificity and plant selectivity while maintaining the proven mechanism of action. Agents which target the isoprenoid pathway in this manner can be expected to be developed as herbicides in the future.

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Pharmaceuticals.

Antimycotics. As in the previous section on agrochemical fungicide development, the enzymes involved in ergosterol biosynthesis have served as targets for pharmaceutical antimycotic development. In this application, the sterol 14α-demethylase has gained prominence as a key enzyme for controlling clinically important pathogenic fungi. Enhanced oral bioavailability, improved pharmacokinetics and greater safety are permitting the broad application of new agents to an increasing patient population. In comparison to older, first and second generation agents, these new compounds appear to be more selective and specific for the fungal sterol 14α-demethylase than for the mammalian counterpart which also shows sensitivity to azole inhibition. Additionally, the new azole antimycotics seem less promiscuous toward other steroid metabolism cytochrome P-450 enzymes which enhances their side-effect profile enormously. These advances serve as a testimonial to the type of progress one can anticipate for other therapeutic agents which target the isoprenoid pathway, and which are currently under development.

Hypocholesterolemic Agents. Elevated serum cholesterol has been shown to be a prominent factor in the etiology of coronary heart disease and atherosclerosis. Thus, the search for safe and effective agents to lower blood cholesterol has dominated pharmaceutical research in recent years. The cholesterol biosynthetic pathway has served as the major focus of this research for obvious reasons. Inhibition of the rate limiting enzyme in the pathway, HMG-CoA reductase, with the statin class of therapeutics has proven to be an extremely effective way to block cholesterol production with ensuing serum cholesterol reduction. Cholesterol biosynthesis blockade at this early step in isoprenoid production, however, also leads to depletion of other key isoprenoids derived from mevalonic acid. Although no obvious, life-threatening toxicities have surfaced with these therapeutics to date, the potential remains. Additionally, despite the high efficacy of the statins, it is necessary to use them in combination with other agents to obtain maximum serum cholesterol lowering in certain patients with extreme hypercholesterolemia. Thus, the hunt continues for more and better agents to block cholesterol formation. The tactics involve directed synthesis toward squalene synthetase inhibitors which would target the cholesterol branch of the isoprenoid pathway specifically. Also, mimicry of naturally occurring oxysterol regulators of HMG-CoA reductase, is proving to be affective in producing novel agents to fight elevated cholesterol levels. One can expect to see pharmaceutical drug discovery providing us with numerous agents to control and manipulate cholesterol production. Many of these agents will find utility in the clinical setting, while others will be valuable tools for the basic scientist to use in dissecting the isoprenoid pathway in greater detail.

NEW HORIZONS

Extension of Pathway Diversity.

Prenylated Proteins. An emerging area of intense biochemical and chemical research was born with the realization that proteins can be modified post-translationally by polyisoprenoid attachment. Protein prenylation serves as an initial step in a processing sequence which includes carboxy terminal proteolysis, prenylcysteine transmethylation, and membrane association. Unique protein prenylation sequences have been defined such as the Caax sequence found in p21^{ras} along with others found in a number of small molecule weight G-proteins and nuclear lamanins. Incorporation of farnesyl or geranylgeranyl groups via highly specific protein prenyl transferase enzymes which recognize the protein prenylation motifs are starting to be described. The potential for therapeutic and agrochemical development which targets these enzymes and processes is sure to be realized in the coming years. It can also be anticipated that new findings will emerge from these studies which will contribute to advances in the areas of natural products, enzymology, biochemistry, and chemistry of the isoprenoid pathway. Thus, the rich tradition of the isoprenoid pathway can be expected to continue with novel advances contributing to our general scientific knowledge for years to come.

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Chapter 2

Regulation of Monoterpene Biosynthesis in Conifer Defense

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Oleoresin is a complex mixture of terpenoids that serves an important role in the defense of conifers against herbivores and pathogens. The regulation of oleoresin monoterpene biosynthesis in a range of conifer species was examined. The levels of constitutive monoterpene cyclase activity were correlated with the monoterpene content of the oleoresin and with the anatomy of the resin-secreting structures. Stem wounding to simulate bark beetle attack resulted in significant increase in cyclase activity in some conifer species, such as *Abies grandis*, but only minor changes in constitutive levels in others, such as *Pinus* species, thus indicating different mechanisms of regulation of oleoresin biosynthesis. The increase in cyclase activity following wounding of *A. grandis* represented the enhancement of constitutive cyclase activities and the appearance of additional distinct enzymes that were absent in unwounded tissue.

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Anatomy of Resin-Accumulating Structures

Conifer oleoresin is a complex mixture of monoterpenes and diterpene resin acids, with lower levels of sesquiterpenes and phenolics. This viscous, odoriferous material with antiseptic properties is accumulated at wound and infection sites, and is considered to be an important component of the defense response (*1*,*2*). Conifers accumulate oleoresin in specialized organs in leaves, roots, stems and seeds (*3*). The anatomical organization of these specialized resin-containing structures in stem tissue differs among species and is considered an important feature for systematic classification (*3*). In some species (i.e., *Thuja plicata*), oleoresin is accumulated in simple "resin cells", scattered unicellular structures that lack any high level of organization. In other conifer types (*Sequoia* and *Abies* species), individual resin cells are present together with multicellular structures in the form of "resin blisters" or "cysts". These sac-like structures, filled with oleoresin, are lined by a single layer of epithelial cells that lignify and die during the year of origin. Other members of the Pinaceae display a much higher degree of organization of the resin-accumulating structures. In *Pseudotsuga* and *Larix* species, resin passages are found together with resin cells. Resin passages are constricted, but interconnected, tube-like structures filled with oleoresin. The

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epithelial cells lining the resin passages are long lived, remaining active for several years. The genus *Pinus* displays the highest degree of morphological sophistication in resin-accumulating structures. Resin cells are absent, and an interconnected array of unconstricted resin ducts exist throughout the wood and bark of the trunk. The epithelial cells lining these ducts remain active for many years. It is presumed that the epithelial cells produce and secrete the oleoresin (*4*), and these specialized cells contain an abundance of leucoplasts that may be involved in resin biosynthesis (*5*).

The Origin of Oleoresin

The terpenoid components of conifer oleoresin (volatile mono- and sesquiterpenes, and non-volatile diterpene resin acids) are biosynthesized via the ubiquitous mevalonate pathway (*6*). Acetyl CoA is transformed in several steps to mevalonic acid that undergoes pyrophosphorylation and decarboxylation to isopentenyl pyrophosphate. The isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate, followed by condensation of these two C_5 units under the influence of prenyltransferase gives rise to geranyl pyrophosphate. Geranyl pyrophosphate serves as the substrate for the monoterpene synthases (cyclases) (Figure 1) and thus represents the key precursor of monoterpene biosynthesis. Geranyl pyrophosphate may also undergo further elongation to farnesyl pyrophosphate (C_{15}) and geranylgeranyl pyrophosphate (C_{20}) by action of the corresponding prenyltransferases, and these intermediates in turn give rise to the sesquiterpenoids and diterpenoids, respectively. Thus, the monoterpene cyclases function at an important branch-point in isoprenoid metabolism in conifers and are likely to play a regulatory role in monoterpene biosynthesis (*6*).

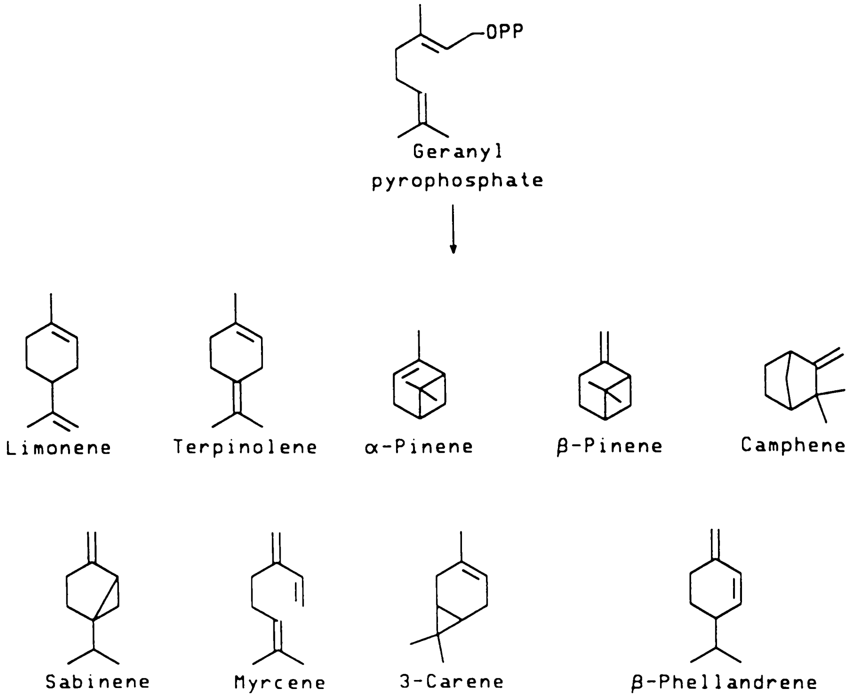

Figure 1. Major olefinic products generated from geranyl pyrophosphate by monoterpene cyclases from conifer species.

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It, therefore, seemed safe to assume that the level of monoterpene cyclase activity measured *in vitro* would accurately reflect the *in vivo* capacity for monoterpene biosynthesis.

Extraction of Monoterpene Cyclases

A method for the efficient extraction of monoterpene cyclase activities from conifer stem tissue was needed; however, such enzyme isolation is complicated since woody tissue is very robust (with a high proportion of lignified, dead cells) and contains high levels of deleterious soluble phenolic materials. Monoterpene cyclase activity could be readily measured in cell-free extracts of frozen (liquid N_2), pulverized pine wood pieces containing the xylem, xylem mother cells, cambium, ray parenchyma and resin ducts, but lacking any bark tissue (phloem) (*7*). Conversely, no activity could be detected in bark extracts despite that this tissue contained abundant resin ducts. Furthermore, bark extracts inhibited the monoterpene cyclase activity of wood extracts (*7*). This inhibition was irreversible, but was efficiently overcome by the addition of polyvinylpyrrolidone to the extracts during homogenization (*7*). Total monoterpene cyclase activity could then be readily determined in cell-free extracts from either wood, bark or whole stems of all conifer species tested when polyvinylpyrrolidone was included in the isolation step. Surprisingly, the cross-linked, insoluble form of the polymer (polyvinylpolypyrrolidone, which is often considered similar in properties to polyvinylpyrrolidone) could not substitute for the soluble form. The browning associated with phenolic polymerization was prevented by polyvinylpolypyrrolidone but enzyme activity was not preserved (*7*).

Survey of Constitutive Monoterpene Cyclase Activity

The assay for monoterpene cyclase activity in conifer extracts involves the divalent metal ion-dependent conversion of ^{3}H-labeled geranyl pyrophosphate to monoterpene olefins that are extracted into hexane or pentane, purified by chromatography on silica, separated and identified by radio-GLC, and quantitated by liquid scintillation counting (*8*). Using this assay, saplings of ten diverse conifer species were surveyed. A direct relationship was determined between the degree of morphological differentiation of the resin-accumulating organs, the level of monoterpene cyclase activity measured *in vitro*, and the level of monoterpenoids present in the oleoresin of the different species (*7*). Species that contain only scattered resin cells (e.g., *Thuja plicata*) and little endogenous oleoresin contained low cyclase activity levels, whereas species that possessed a higher degree of organization of the resin-accumulating organs and copious oleoresin (e.g., *Pinus ponderosa*) displayed up to 200-fold higher constitutive cyclase activity. These observations support the assumption that the level of monoterpene cyclase activity reflects monoterpene biosynthetic capacity in the various conifer species.

Effects of Wounding on Monoterpene Biosynthesis

Oleoresin is accumulated at wound and infection sites, and it is often assumed that this resin is synthesized locally in response to such stresses (*9*). The possibility that the accumulated resin is simply translocated to the wound site has been largely overlooked; however, the observation that resin appears at wound sites within seconds after wounding suggests that, at least in some species (such as larches and pines), resin can be translocated via the interconnected system of passages or ducts. Based on these considerations, two types of resinosis have been described (*10*). Primary (constitutive) resin is that which is formed continuously and accumulates in the specialized secretory structures, and secondary (induced) resin is that which is formed on wounding (or fungal infection) and is generally thought to be produced in tissues that usually do not synthesize resin. To assess the reliance of different conifer species upon primary or secondary resinosis in defense, monoterpene cyclase activity was determined in extracts from wounded trees (seven days after wounding) and compared to the corresponding extracts from nonwounded controls (*11*) (Table I).

In general, higher levels of monoterpene cyclase activity were found in extracts from wounded tissue relative to nonwounded control stems. The most significant increases in activity following wounding were found in *Abies grandis* (14-fold) and *Picea pungens* (5-fold) stems. The response observed was highly dependent on the species tested. *Thuja plicata*, which possesses only resin cells and accumulates low levels of resin, displayed a very low (but significant) increase in cyclase activity after wounding. *Pinus contorta* and *P. ponderosa*, species that exhibit a relatively high level of constitutive cyclase activity and possess a very intricate system of interconnected resin ducts, did not evidence a statistically significant increase in cyclase activity seven days after wounding. Thus, although oleoresin production is an important defense mechanism in conifers, the relative importance of primary versus secondary resinosis varies among species. The accumulation of resin at wound sites of pines is likely to be largely of the "primary" type (i.e., preformed and transported to the site of injury through the duct system), at least during the first week after wounding, whereas grand fir (that lacks a duct or passage system) seemingly relies almost exclusively on "secondary" resinosis as a defense response.

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Grand Fir as a Model for the Study of Wound-Induced Oleoresinosis

As grand fir stems displayed the greatest wound-dependent increase in cyclase activity, this response was studied in more detail. Increases in cyclase activity were noted as soon as one day following wounding, rising to ten-fold above control levels nine days afterward (*11*). Both wood (containing cambium and xylem-mother cells) and bark (from the cambium layer outward) tissues evidenced enhanced cyclase activity. The wound-induced increase was restricted to a few cm surrounding the wound site (Figure 2A); however, tissue throughout the length of the stem had the potential for this wound-induced response (Figure 2B). Many forms of wounding (i.e., pin-pricking, razor cuts, bending, etc.) elicited the response, the intensity of which was dependent on the severity of the challenge applied (*11*). All of the experiments reported above were performed with two-year-old saplings grown under greenhouse conditions (*11*), and the wound-induction of monoterpene cyclase activity was confirmed in mature grand fir trees (~ 30 year old) growing in a natural stand on the Umatilla National Forest (Oregon) by sampling tissue around wound sites one and two weeks after wounding (unpublished). The induction of monoterpene biosynthesis upon wounding provides grand fir with the ability to accumulate copious resin at the wound site without the need for a prefilled grid of resin ducts such as those of *Pinus* species.

The monoterpene olefins generated from [^{3}H]geranyl pyrophosphate in crude cell-free extracts from wounded and nonwounded stems were analyzed by radio-GLC (*12*), and the results (Figure 3) indicated that the same spectrum of products were generated by both preparations. However, the products of the wound-induced cyclization activity were notably enriched in pinenes (18% α-pinene [43-fold increase], 24% β-pinene [32-fold increase], 24% limonene [7-fold increase], 29% other olefins [8-fold total increase]). Further purification of the cyclases from extracts of wounded and control stems revealed that this difference in product distribution was apparently due to the enhancement of existing activities as well as to the appearance of new cyclization activities absent before wounding (*12*). Total cyclase activities in crude extracts from wounded

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Table I. Constitutive and Wound-Inducible Monoterpene Cyclase Activity in Several Conifers

	Monoterpene Cyclase Activity	
Species	*Control (constitutive)*	*Wounded (induced)*
	pmol (h•mg fresh wt)-1	
Thuja plicata	0.01 ± 0.005	0.06 ± 0.01*
Sequoia sempervirens	0.07 ± 0.02	0.11 ± 0.02
Abies grandis	0.24 ± 0.09	3.30 ± 0.54*
Abies concolor	1.73 ± 0.18	3.34 ± 0.71
Abies lasiocarpa	2.70 ± 0.58	2.52 ± 0.63
Pseudotsuga menziesii	2.11 ± 0.54	2.61 ± 0.55
Larix occidentalis	6.41 ± 1.14	8.56 ± 0.75
Picea pungens	3.78 ± 0.83	13.5 ± 0.77*
Pinus contorta	8.43 ± 1.34	10.2 ± 1.00
Pinus ponderosa	14.5 ± 1.27	16.1 ± 2.11

Saplings were wounded along the entire stem by horizontal slicing with a razor blade and monoterpene cyclase activity was determined seven days after wounding. Control saplings were untreated and maintained under identical conditions. The mean and standard error from five determinations are reported, and an asterisk indicates that the difference between wounded and control samples is significant by the Duncan *t* test with $\alpha = 0.05$. SOURCE: Adapted from ref. *11*.

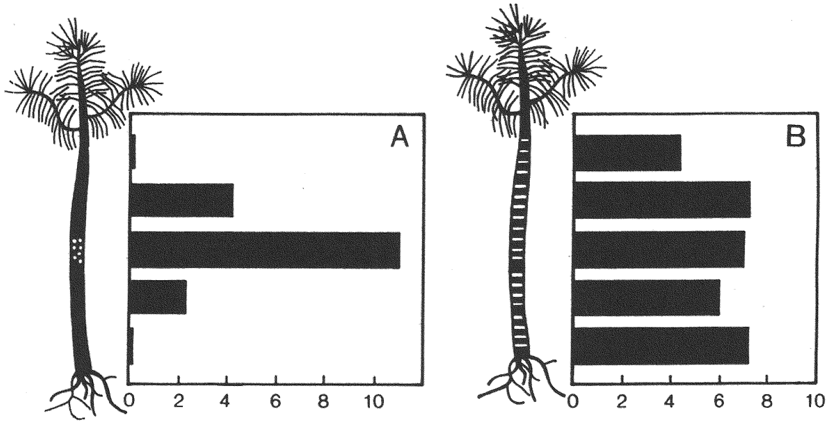

Figure 2. Localization of wound-inducible resinosis in proximal and distal stem sections of grand fir. Trees were wounded in the midstem (A) or along the entire length of the stem (B), and monoterpene cyclase activity was determined in the corresponding stem sections after seven days. The fold increase in cyclase activity is relative to the equivalent stem sections from nonwounded controls. SOURCE: Adapted from ref. 11.

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and nonwounded trees were partially purified by anion exchange and hydrophobic interaction chromatography before fractionation by high resolution anion exchange chromatography on a Mono Q column (FPLC) (Figure 4). Several distinct cyclase activities were resolved, some of which were present in the control preparations but greatly enhanced in wounded tissue extracts (peak II), and others that were virtually absent in controls but which were very prominent in preparations from wounded tissue (peaks I, and III to VI in Figure 4). Subsequent radio-GLC analysis of the products generated by selected Mono Q fractions established that the cyclase activities separated by this technique produced different olefinic products from geranyl pyrophosphate (*12*). Thus, activity I produced mainly sabinene (34%), terpinolene (27%), limonene (16%) and other olefins (23%), whereas activity II (the principal cyclization activity in control stems) was greatly enhanced after after wounding and generated largely limonene (~ 50%) with lower levels of terpinolene (~ 14%) and other olefins (36%). Although this observation would seem to suggest that limonene should be an abundant component of grand fir oleoresin, it in fact comprises less than 5% of the monoterpene fraction (7). There are several possible explanations for this apparent discrepancy. It is conceivable that the extraction or assay conditions abnormally favor the formation of limonene *in vitro*, by a monoterpene cyclase or

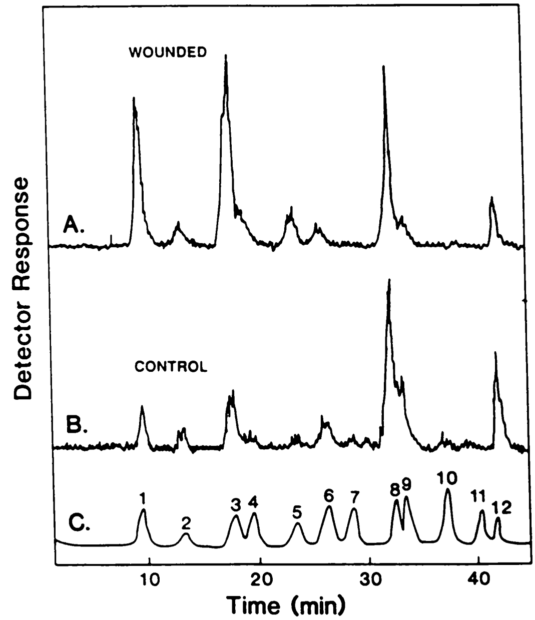

Figure 3. Radio gas-liquid chromatographic separation of the biosynthetic products generated from [1-^{3}H]geranyl pyrophosphate by crude cell-free extracts from wounded grand fir stems (A) and from nonwounded control stems (B). Assay conditions, product isolation, and column conditions have been described (12). The smooth *lower tracing* (C) is the thermal conductivity detector response to authentic standards of α-pinene (1), camphene (2), β-pinene (3), sabinene (4), 3-carene (5), myrcene (6), α-terpinene (7), limonene (8), β-phellandrene (9), γ-terpinene (10), *p*-cymene (11), and terpinolene (12). The *upper tracings* (A and B) are the radioactivity recorded by the Packard 894 monitor attached to the chromatograph. SOURCE: Reprinted with permission from ref. *12*. Copyright 1991.

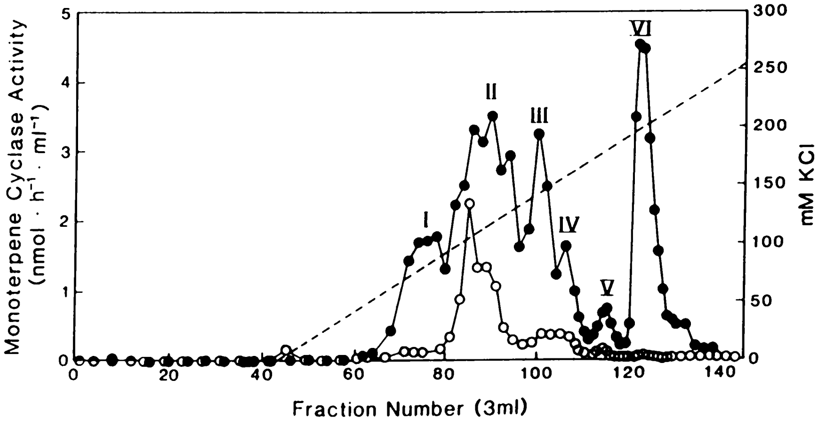

Figure 4. Gradient elution anion-exchange chromatography on Mono Q (FPLC) of partially purified cell-free extracts from grand fir stems. Total monoterpene synthase activity in preparations from wounded stems (●——●) and from nonwounded controls (○——○) is plotted. Roman numerals indicate distinct synthase activities as described in the text. SOURCE: Reprinted with permission from ref. *12*. Copyright 1991.

other enzyme. Alternatively, it is possible that limonene is produced at significant rates *in vivo* but is subsequently degraded or emitted to the atmosphere. Emission of monoterpenes from conifers is well-documented (*13*). Experiments designed to address this apparent anomaly are under way.

Eluting after the limonene cyclase (peak II) are four distinct cyclization activities: peak III, generating predominantly 3-carene; peak IV, producing predominantly β-pinene; peak V, yielding largely the acyclic olefin myrcene; and finally, peak VI, generating a 3:2 mixture of β-pinene and α-pinene. This last enzyme to elute from the Mono Q column is the principal wound-inducible cyclase, and it was purified to homogeneity and identified as a 62 kDa polypeptide as outlined below. The enzyme generated only the (-)-enantiomers of α- and β-pinene, and both of these pinene products were confirmed to be synthesized by the same protein from a common intermediate using isotopically sensitive branching experiments (*14*).

Identification of the Wound-Inducible Pinene Cyclase

Since the pinene cyclase was the most abundant monoterpene synthase in wounded tissue, but was seemingly absent in uninjured grand fir stems, this enzyme was examined in greater detail. The corresponding partially purified preparations from wounded and control trees were first systematically compared in an attempt to identify the pinene cyclase. Although the enzyme was well-resolved from other cyclases by the anion exchange chromatography step (peak VI, Figure 4), the low abundance of the protein and the complexity of the mixture required an additional purification step by native PAGE before a difference between the protein profiles of the samples could be observed. At this state of purity, the major protein present in preparations from wounded stems was a 62 kDa species (by SDS-PAGE analysis, Figure 5). This protein was absent in preparations from nonwounded plants, suggesting that the wound-inducible 62 kDa protein was the (-)-pinene cyclase (*12*).

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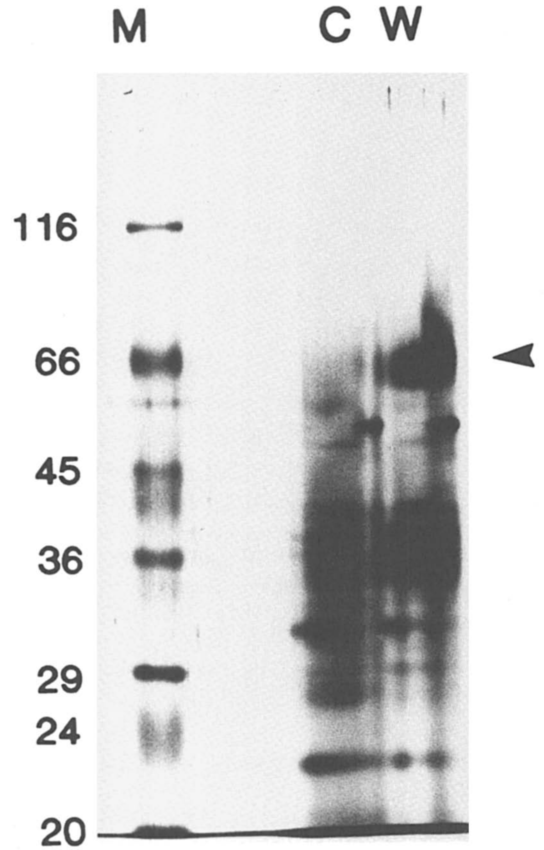

Figure 5. SDS-PAGE of the partially purified (-)-pinene cyclase from wounded grand fir stems following the Mono Q chromatography step (peak VI from Figure 4) and non-denaturing PAGE (W) (5 μg total protein). The corresponding protein fraction (~ 2 μg total protein) from preparations of nonwounded controls (devoid of (-)-pinene cyclase activity) was run in parallel (C). The silver stained proteins on this SDS gel appear as spots rather than bands because the direction of migration is perpendicular to that of the first (native) gel. The migration of protein standards (in kiloDaltons) is also indicated (M). SOURCE: Reprinted with permission from ref. *12*. Copyright 1991.

The pinene cyclase was recently purified to homogeneity using anion exchange (DEAE-cellulose) and hydrophobic interaction chromatography (phenyl Sepharose) followed by high resolution anion exchange on a Mono Q column (FPLC) and native PAGE (at neutral pH) and, finally, renaturing of activity after SDS-PAGE (*15*). The pure protein was employed to obtain monospecific polyclonal antibodies against the cyclase (*16*) using the whiffle-ball technique in rabbits (*17*). The polyclonal antibodies thus generated recognized the 62 kDa protein antigen by Western blotting (Figure 6), and this protein was shown to be present only in extracts from wounded stems. Thus, it is clear that the enhanced level of pinene cyclase activity observed after wounding is the result of an increase in the amount of cyclase protein.

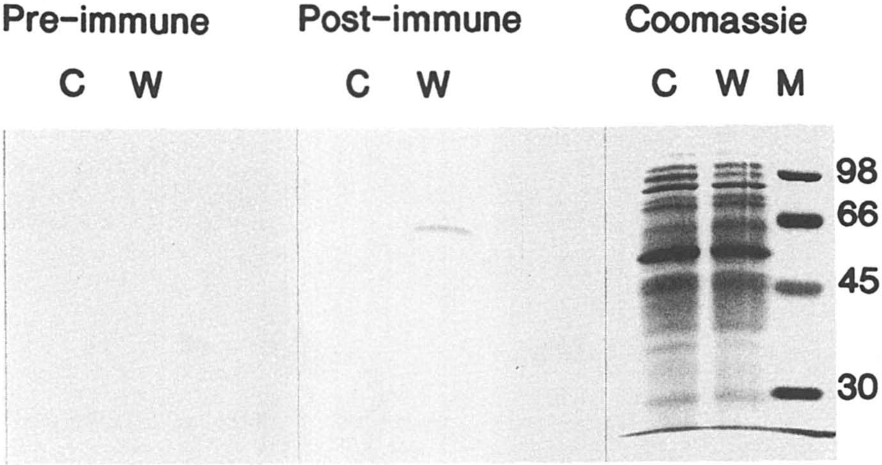

Figure 6. Immunoblotting of grand fir stem extracts. Cell-free preparations from wounded (W) and control (C) stems were purified by DEAE-cellulose chromatography, separated by SDS-PAGE, and electroblotted onto PVDF. The 62 kDa cyclase protein was detected using alkaline phosphatase conjugated to goat anti-rabbit IgG as described elsewhere (16). A total protein stain and molecular weight markers are also shown. SOURCE: Adapted from ref. 16.

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Conclusion

The results described here indicate that grand fir, which possesses relatively little constitutive resin, relies on wound-inducible oleoresinosis in defense. This property contrasts with other gymnosperms (such as the pines) which possess high levels of constitutive resin that is translocated to the wound site, but which undergo little change in biosynthetic capability in response to wounding (at least within the first few days of injury). The control of resin biosynthesis thus differs significantly between conifer species. There also appears to be several levels of regulation of oleoresin production within grand fir. Thus, a low level of cyclization capability is constitutively present in intact tissue, presumably associated with the epithelial cells lining the resin blisters. On wounding, total cyclization activity is greatly enhanced which is the result of both the apparent increase of constitutive activities and the appearance of new activities derived by synthesis of new cyclase protein.

The induction of other terpene cyclases in plant tissues has been described, including the elicitor-mediated induction of a sesquiterpene cyclase in *Nicotiana tabacum* cell cultures (*18*) and the fungal and UV light induction of diterpene cyclases in *Ricinus communis* and rice (*19,20*). The observed induction of monoterpene cyclase activity in grand fir differs from these and other phytoalexic responses (*18-22*) in several ways: many different monoterpene cyclases are induced in grand fir; wounding alone is sufficient to induce the response; and the induced compounds are antimicrobial/insecticidal metabolites of a type already present in unchallenged tissue (*1, 2*).

Acknowledgments

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Chapter 3

Natural Rubber Biosynthesis

A Branch of the Isoprenoid Pathway in Plants

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Natural rubber is a plant product in high demand in the United States. Chemically, natural rubber is a high molecular weight hydrocarbon polymer largely composed of isoprene monomers linked, in a head-to-tail fashion, to form long chains of cis-1,4-polyisoprene. Simply stated, the longer the molecule, the better the performance characteristics of the rubber. Synthetic rubber cannot substitute for natural rubber in applications that require high elasticity, resilience and minimal heat build-up (e.g., high performance tires, latex gloves). Natural rubber is often blended with synthetic forms to confer desirable high performance characteristics. Synthetic rubber is derived from petroleum products and so is a non-renewable resource; natural rubber should be available indefinitely from renewable plant sources. At present, the United States imports over 1,000,000 tons of natural rubber each year, at a cost of approximately $1,000,000,000, to support its commercial and defense needs. Natural rubber constitutes over 40% of the total amount of both natural and synthetic rubber used and is expected to increase its market share substantially over the next few years, leading to an even greater demand. However, virtually all natural rubber is obtained by tapping the Brazilian rubber tree (Hevea brasiliensis), a tropical species. Even if H. brasiliensis could be grown in some developed countries, the cultivation and harvesting costs would be prohibitive. Consequently, developed countries, and temperate regions of the world, are wholly dependent on nondomestic sources of this vital raw material. The capacity for rapid expansion of domestic natural rubber production would protect the United States against emergency shortages induced by crop failure in the H. brasiliensis plantations or by changing political climes. The strategic necessity of rubber is,

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of itself, sufficient incentive to develop a domestic rubber-production system, even if it lacked commercial competitiveness at the time of its completion. Nevertheless, crop costs will certainly rise as the producing countries develop further. The prospects of commercial competitiveness of domestic rubber will steadily improve as natural rubber prices increase.

Our research is directed towards the development of a domestic natural rubber supply for the United States. To achieve this goal we have decided upon a biotechnological approach: to this end we need to identify and isolate the enzymes and genes responsible for the cis-1,4-polymerization of isoprene unique to rubber-producing plants. Of course, we must also clarify the biochemistry of rubber formation and understand the in vivo functioning of the enzymes and genes involved in natural rubber biosynthesis. Once this is done, it should be possible to isolate, then insert and express, the appropriate genes in annual plants and, perhaps, in micro-organisms, and then optimize these systems to produce high quality natural rubber.

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Sources of Natural Rubber

Natural rubber is synthesized and accumulated in more than 2,000 species of plants from about 300 genera, as well as in at least two fungal genera (4,6). The high quality rubber produced by H. brasiliensis has a mean molecular weight of about 1,500,000 Da. Parthenium argentatum A. Gray (guayule) also produces high molecular weight rubber. However, most rubber-producing plants make much shorter rubber chains of $\leq$ 50,000 Da. Rubber yield can also vary greatly from species to species. This wide range of available material can be exploited when addressing different aspects of the development of a domestic rubber resource. A highly developed plant such as H. brasiliensis, that forms its rubber in a pipe-like network of laticifers running beneath the bark (3) may prove to be the best experimental material for studying the biochemistry of rubber biosynthesis; a species such as P. argentatum, that produces rubber in individual parenchyma cells (6), may provide the simplest genetic system.

Biosynthesis of Natural Rubber

Natural rubber is produced by the action of the enzyme rubber transferase (RuT) (EC 2.5.1.20) (6) on the substrates isopentenyl pyrophosphate (IPP) and allylic pyrophosphates, in a side-branch of the ubiquitous isoprenoid pathway (Figure 1). RuT, a prenyl transferase, requires divalent cations (including Mg^{2+} or Mn^{2+}) for activity, but RuT, IPP (the substrate for

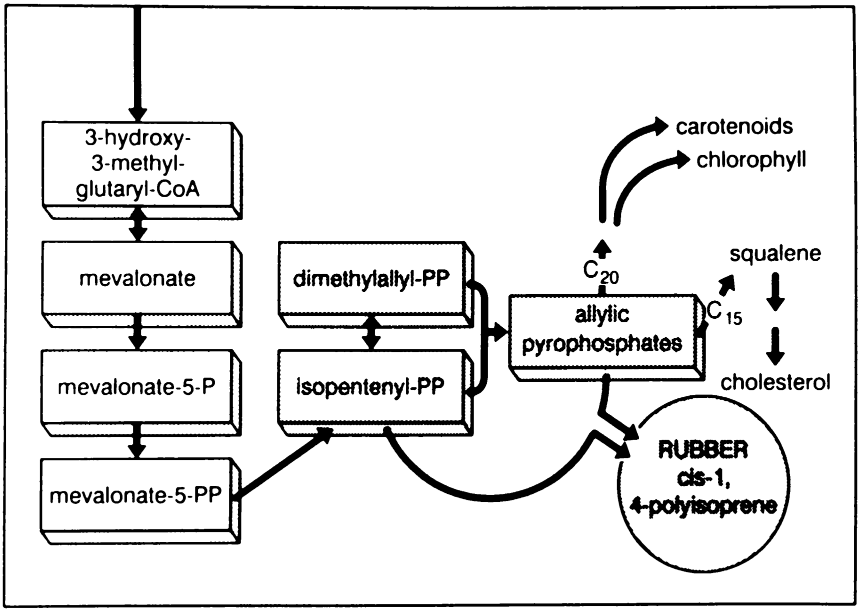
3-hydroxy-3-methyl-glutaryl-CoA
mevalonate
mevalonate-5-P
mevalonate-5-PP
dimethylallyl-PP
isopentenyl-PP
allylic pyrophosphates
C20
C15
carotenoids
chlorophyll
squalene
cholesterol
RUBBER cis-1, 4-polyisoprene

Figure 1. The isoprenoid pathway, illustrating the position of natural rubber biosynthesis.

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elongation) and Mg^{2+} together will not result in rubber formation: a second substrate, an allylic pyrophosphate, is needed to initiate the polymerization process. A number of allylic pyrophosphates are capable of initiating rubber formation in isolated washed rubber particles (5). It has been reported that the efficiency of initiation increases with the chain length of the initiators ($C_5 < C_{10} < C_{15} \leq C_{20}$; 5,17), although it is unclear how this effect is mediated. The enzymatic steps most intimately concerned with rubber biosynthesis are shown in Figure 2, and entail the isomerization of IPP to dimethylallyl pyrophosphate (DMAPP), and the synthesis of C_{10}, C_{15} and C_{20} allylic pyrophosphates (in a trans configuration) from IPP (non-allylic C_5) and DMAPP (allylic C_5), before RuT can form cis-1,4-polyisoprene from IPP.

Hence, three biochemical stages are involved in rubber molecule formation: (1) **initiation**, which requires an allylic pyrophosphate starter molecule; (2) **elongation**, which is the polymerization into rubber of isoprene units from isopentenyl pyrophosphate (IPP); and (3) **termination**, the release of the chain from the RuT enzyme, and which may or may not be a dedicated enzymatic process.

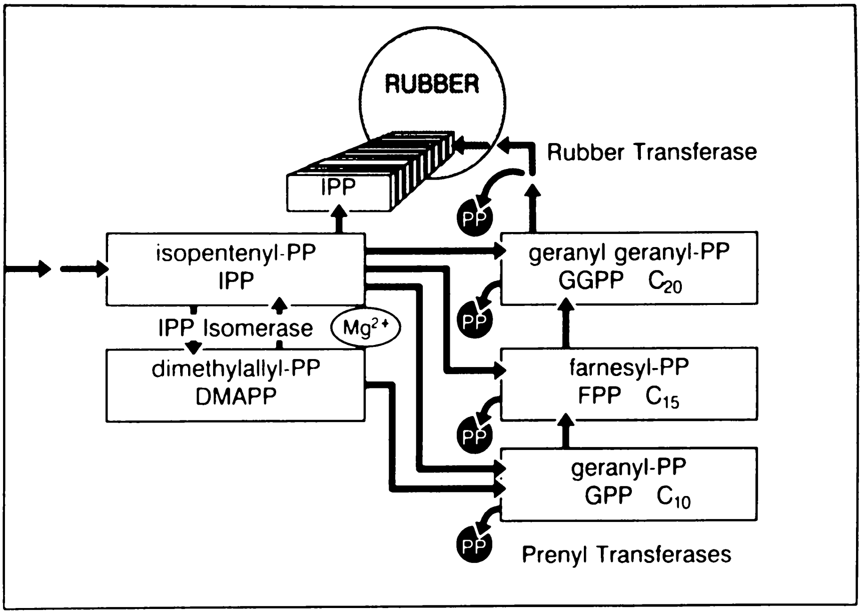
RUBBER
Rubber Transferase
IPP
isopentenyl-PP
IPP
IPP Isomerase
Mg2+
dimethylallyl-PP
DMAPP
geranyl geranyl-PP
GGPP C20
farnesyl-PP
FPP C15
geranyl-PP
GPP C10
PP
PP
PP
PP
Prenyl Transferases

Figure 2. Natural rubber biosynthesis from isopentenyl pyrophosphate.

For commercial success, production of high-grade natural rubber requires a system that produces many long-chain rubber molecules as, generally speaking, the longer the polyisoprene chain the better the high-performance characteristics of the rubber. Nevertheless, short-chain rubber producers, such as Ficus elastica provide excellent systems for investigating chain termination as they have a much higher ratio of termination events to elongation events than do long-chain producers like H. brasiliensis or P. argentatum. The biochemistry of the termination mechanism has not been thoroughly investigated, although it has been shown that the terminal pyrophosphate can be hydrolyzed, esterified or cyclized depending on the species and conditions (Tanaka, 1989).

The isolation of RuT and its transfer and expression in other systems is probably insufficient in itself to achieve a new rubber supply. Among many possibilities, other parts of the isoprenoid pathway (Figure 1) may need to be examined in order to optimize the production of endogenous substrates for rubber production in micro-organismal and plant biosynthetic systems, and also to

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prevent detrimental substrate deficiencies further along the isoprenoid pathway. For example, the isoprene monomers are derived from isopentenyl pyrophosphate (IPP), which is synthesized from carbohydrates via acetyl-CoA, HMG-CoA and mevalonate (Figure 1). The production of mevalonate by the enzyme HMG-CoA reductase may be a rate-limiting step in *H. brasiliensis* rubber production (13). Considerable progress has already been made as the *H. brasiliensis* farnesyl pyrophosphate (FPP) synthetase (12) and the FPP-synthetase (2) and IPP-isomerase (1) from yeast have already been characterized.

Localization of Rubber Transferase

Rubber is compartmentalized into cytoplasmic rubber particles both in laticiferous species such as *H. brasiliensis* (3), and in species that produce rubber in parenchyma cells such as in the bark of *P. argentatum* (6). These two species are unusual among rubber-producers as they both can make commercial-grade rubber.

During rubber biosynthesis, isopentenyl pyrophosphate is obtained from the aqueous environment outside the rubber particles and is dephosphorylated and polymerized by the RuT enzyme; the developing isoprene polymers extend into the particle interior. The formation of rubber can be assayed by following the incorporation of labelled isoprene from ^{14}C-IPP into newly-synthesized chains. In order to isolate the RuT enzyme it is first necessary to determine the location of the enzymatic reaction. The enzymes responsible for rubber biosynthesis may or may not be attached to the particles.

Rubber particles of *H. brasiliensis* and *P. argentatum*, isolated by using a centrifugation/flotation procedure (10), had high rubber biosynthetic activity, as assayed by the incorporation of radiolabel from ^{14}C-IPP into rubber with FPP as the initiator molecule. However, no isoprene was incorporated in the absence of allylic pyrophosphates. In common with other isoprenoid enzymes, the reaction requires divalent cations, probably Mg^{2+} *in vivo*. Repeated washing of *P. argentatum* rubber particles, using the flotation/centrifugation procedure, did not reduce the level of the highly active bound rubber transferase when assayed in the presence of IPP (elongation substrate) and FPP (initiation substrate) (10). Similar experiments have demonstrated that most, if not all, of the RuT activity in *H. brasiliensis* whole latex (latex tapped from laticifers and from which no constituents have been removed) can also be accounted for by an enzyme firmly attached to the washed rubber particles (WRPs; purified particles from which the other constituents of whole latex have been removed) (Cornish, K., USDA-ARS, unpublished results). Expressed as a function of the number of rubber particles assayed, no

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reduction of RuT activity with increasing purification of rubber particles was observed for either species. Thus, the RuT molecules are firmly associated with rubber particles in both *H. brasiliensis* (4,9) and *P. argentatum* (8,10).

However, Light and Dennis (12) determined that the cytosolic fraction of *H. brasiliensis* whole latex was essential for rubber biosynthesis by Sephacryl S-300 gel-filtered particles when assayed in the presence of ^{14}C-IPP. This led them to believe that, instead of a particle-bound enzyme, a soluble RuT was present in this laticiferous species. (It is not possible to do the comparable experiment with *P. argentatum* as the particles are prepared from a bark homogenate, not an intact latex.) Their conclusion (12) was consistent with early work where a soluble protein extract from *H. brasiliensis* latex was thought to catalyze the extension of pre-existing rubber pyrophosphate chains in WRPs (4) and a soluble "rubber transferase" was reported by McMullen and McSweeney (15). Nevertheless, none of these soluble extracts could synthesize rubber in the absence of rubber particles. Also, in the presence of WRP, they could be replaced by a series of allylic pyrophosphate initiator molecules (5,9,14). Hence, it is likely that these proteins form part of the initiating system, synthesizing allylic pyrophosphates essential to the initiation of new rubber molecules, rather than polyisoprene formation itself. Light and Dennis (12) went on to isolate a soluble prenyl transferase (PT; monomer, 38 kD) as the active component from the latex cytosol. They found that this enzyme behaved, in solution, as an FPP-synthetase, as I have also confirmed (Cornish, K., USDA-ARS, unpublished results; purified PT was a generous gift from Genentech). Nevertheless, in the presence of gel-filtered particles, IPP, DTT and Mg^{2+} this PT induced the production of rubber. In similar experiments, WRP from *H. brasiliensis* latex assayed in the presence of IPP and Mg^{2+} had a much lower rate of IPP-incorporation than whole latex (Cornish,K., USDA-ARS, unpublished results). Whole latex activity could be partially restored to the WRP by the addition of PT. However, when the WRP were assayed in the presence of FPP as well as IPP, the bound RuT supported much higher rates of IPP-incorporation than whole latex, or WRP plus prenyl transferase.

The Role of Prenyl Transferase in Rubber Biosynthesis

An alternative explanation to account for the observed stimulatory effect of the prenyl transferase on rubber biosynthesis, other than it actually being RuT, was still left open. Could PT be supplying the source of allylic pyrophosphate necessary to initiate *de novo* rubber biosynthesis? This seems likely because, as discussed earlier, PT behaves as an FPP-synthetase in the absence

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of rubber particles (12), and FPP is a potent initiator (5,9,10,14).

If PT is stimulating rubber formation by synthesizing FPP initiator molecules, then this presupposes the presence of either DMAPP or IPP-isomerase in the *in vitro* assay (see Figure 2): prenyl transferases cannot make allylic pyrophosphates in the absence of DMAPP. Using a specific inhibitor of the isomerase (3,4-oxido-3-methyl-1-butyl diphosphate; 16), kindly supplied by C. Dale Poulter (University of Utah), considerable IPP-isomerase activity was detected in whole latex and also a low level of contamination in the PT preparation itself (Cornish, K., USDA-ARS, unpublished results). As only one initiator molecule is required for each rubber molecule, made from perhaps thousands of isoprene molecules, a very small amount of DMAPP or IPP-isomerase in a rubber particle preparation would permit the synthesis of enough FPP to allow a considerable production of rubber.

Of course, the demonstration of a role of the Light and Dennis (12) PT in rubber molecule initiation does not rule out an additional role in rubber elongation. Examining the effect of PT in the presence of different concentrations of FPP (the substance made by PT in solution) should reveal any additional role. PT had no effect on IPP-incorporation by whole latex even at saturating FPP concentrations (Cornish, K., USDA-ARS, unpublished results). The possibility that this negative result was caused by a saturating level of PT already present in whole latex was discounted as the [FPP]-dependence of IPP-incorporation by whole latex was identical to that of WRP (from which the soluble components of whole latex, including PT, have been removed) (Cornish, K., USDA-ARS, unpublished results).

Particle-Bound Rubber Transferase

The kinetics of IPP-incorporation by *P. argentatum* WRP (10) are straight-forward concentration dependencies on both IPP and FPP. Both substrates give rise to linear Eadie-Hofstee plots which are indicative of single enzyme systems (10). Similar experiments using *H. brasiliensis* WRP demonstrated that this RuT behaves, biochemically, in a manner virtually identical to the *P. argentatum* RuT. The apparent K_m for FPP ranges between 0.8 and 3 uM, for IPP between 250 and 500 uM, and for the cofactor Mg^{2+} between 70 and 80 uM. These biochemical studies suggest that single enzymes are responsible for rubber molecule initiation by FPP and polymerization of IPP. Also, it seems probable that the two processes are catalyzed by a single enzyme although an enzyme complex has yet to be ruled out. If a complex does exist, the active sites responsible for the two processes must be spatially adjacent.

Protein analysis of WRP has been attempted in efforts to distinguish the true RuT enzyme from other particle-bound proteins. Despite the biochemical similarity of the H. brasiliensis and P. argentatum rubber biosynthetic systems, the WRP proteins proved quite distinct. Silver-staining of one-dimensional SDS-PAGE gels revealed that H. brasiliensis WRP possess at least 17 different proteins (Cornish, K., USDA-ARS, unpublished results) whereas P. argentatum WRP have only 4-8 (7).

The P. argentatum 48.5 kD glycoprotein, which is the most abundant rubber particle protein in this species (7) may be RuT itself (8). This protein has been characterized (7) and, at this time, most of its gene has been isolated (Backhaus, R.A., Arizona State University, personal communication, 1991). Experiments indicate that this protein is located largely within the rubber particle, but at the surface with the glycosylated end protruding into the cytoplasm (7). This is an appropriate location for RuT, which must polymerize a hydrophobic polymer to the particle interior while obtaining hydrophilic substrates from the cytosol.

The most abundant particle-bound protein in H. brasiliensis rubber particles is the 14.6 kD "rubber elongation factor" polypeptide (11). This 14.6 kD polypeptide may play a role in rubber biosynthesis although this has not been confirmed. Nevertheless, polyclonal antibodies raised against it did, in one experiment, inhibit RuT activity in whole latex (11).

Conclusions

Rubber particles isolated from H. brasiliensis and P. argentatum synthesize new rubber when incubated with IPP, FPP and Mg^{2+}. The activity of RuT is not diminished by repeated washing, demonstrating that the enzyme is firmly bound to the rubber particle in both species. Also, biochemical studies of rubber biosynthesis by isolated rubber particles of H. brasiliensis and P. argentatum suggest that the two biosynthetic systems are extremely similar. Evidence suggests that the P. argentatum RuT may be the abundant 48.5 kD particle-bound glycoprotein. Which of the H. brasiliensis particle-bound proteins is RuT has yet to be determined.

A considerable mass of information has been accumulated on the biological mechanism of rubber biosynthesis. This has included the isolation and cloning of genes for enzymes involved in the production of allylic pyrophosphate initiators for new rubber molecule. The definitive isolation of the rubber transferase enzyme responsible for rubber molecule elongation, and then its gene, is the final step required before transformation experiments on potential domestic rubber-producing species can be realistically begun.

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Chapter 4

Production of Natural Carotenoids

Influence and Impact of Biotechnology

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Carotenoids are final biosynthetic end-products of isoprenoid synthesis. They occur ubiquitously in nature as colorants and photosynthetic accessory and protective pigments. Carotenoids have long been used as food and cosmetic colorants and as a source of vitamin A. During the past decade, carotenoids have emerged as potential anticarcinogens and radio-protective agents. As a result, the demand for carotenoids is increasing and new methods are being developed for their production. These include improvements in traditional chemical synthesis and new, mass culture production of natural carotenoids from algae, yeast, fungi and recombinant DNA systems. Besides genetic manipulation, new chemical regulators of carotenoid biosynthesis and isolated/immobilized enzyme systems are being developed for carotenoid production.

Carotenoids are a ubiquitous and diverse class of natural pigments, functioning in plants and microbes as photoprotective agents, as photosynthetic accessory pigments and as membrane stabilization components. In animals, carotenoids can function as a source of vitamin A and other retinoids or, as indicated by studies beginning in the 1970s, photoprotective and cancer prevention agents (*1-3*). Carotenoids are also indicated as protectants against lethal irradiation (*4,5*). These protective functions of carotenoids appear due to their ability to quench free radicals and singlet oxygen, acting as antioxidants and protecting cells from oxidative damage (*6*).

Carotenoids may contain from 30 to 50 carbon atoms (Figure 1). The most ubiquitous carotenoids, the 40 carbon tetraterpenoid carotenoids, are synthesized by condensation of two molecules of geranylgeranyl pyrophosphate (*7*). In some microbes, additional isopentenyl groups may be added onto the basic tetraterpenoid backbone to produce carbon 45 and carbon 50 homo-carotenoids. The tetraterpenoid carotenoids may also be oxidized to carotenoids containing less than 40 carbons,

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the apo-carotenoids. The carbon 30 triterpenoid carotenoids have been found only in non-photosynthetic bacteria. While it was initially thought that these unique pigments were degradation products of tetraterpenoid carotenoids, biosynthetic and chemical studies have shown that they are synthesized from two molecules of farnesyl pyrophosphate, similar to squalene and sterols (*8*).

Applications of Carotenoids

The primary use of carotenoids has been as colorants for foods, animal feeds, pharmaceuticals and cosmetics. In 1990, the world market for carotenoids was approximately $120 million (*9*). Of this total, one carotenoid, β-carotene, represented about 60% of the total market or approximately $70 million. This market for β-carotene was divided among applications as a food/feed colorant (65%), vitamin supplement (30%), pharmaceutical/cosmetic colorant (3%) and therapeutic for light-sensitive diseases (2%).

The most commonly used carotenoids for commercial applications are shown in Figure 2. β-Carotene, β-apo-8'-carotenal and canthaxanthin are approved for food use in the U.S. and other countries and their primary application is as food colorants. Lutein and zeaxanthin are used primarily poultry feed supplements, producing the desirable yellow color to the flesh of poultry that consumers demand. Astaxanthin is used in fish feeds to color the flesh of commercial salmon and trout and of decorative carp and other fish prized in the orient.

The production and consumption of carotenoids may be poised for a rapid increase if studies now underway confirm that there is a direct linkage of carotenoids to cancer prevention. In the early 1980s, attention began focussing on the dietary role of carotenoids such as β-carotene in human cancer protection (*10*). Laboratory and human intervention trials have since shown that there are correlations between certain types of cancer and carotenoids. For example, smoking lowers blood carotenoid levels and correlates with lung cancer incidence (*11*), β-carotene and canthaxanthin enhance both specific and non-specific immune function (*12*), and canthaxanthin may inhibit N-methyl-N-nitrosourea-induced breast cancer (*13*). There remains a need, however, for additional studies on not only carotenes and canthaxanthin and cancer protection, but on other carotenoids which are more abundant in foods such as lutein.

The apparent protective function of carotenoids against harmful radiation could also dramatically lead to their increased production and consumption. Protection against lethal X-irradiation by carotenoids such as lycopene and β-carotene was first demonstrated in animal systems in 1959 (*4*). Later studies showed that carotenoids such as phytoene, β-carotene and canthaxanthin can protect against the harmful effects of UV-B light including sunburn and skin tumors (*14,15*). In studies comparing the protective effects of known radioprotecting agents and carotenoids in a standard mouse X-ray lethality assay, we found that carotenoides such as crocetin and bixin were as effective or more effective as radioprotectants than cysteine and urea-based radioprotectants (*5,16*), without the harmful side-effects of the latter compounds.

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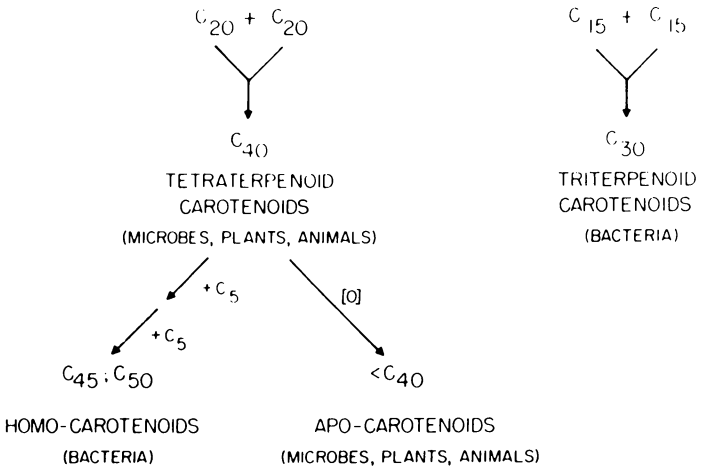
C20 + C20
C40
TETRATERPENOID CAROTENOIDS
(MICROBES, PLANTS, ANIMALS)
+C5
+C5
[O]
C45 ; C50
HOMO-CAROTENOIDS
(BACTERIA)
<C40
APO-CAROTENOIDS
(MICROBES, PLANTS, ANIMALS)
C15 + C15
C30
TRITERPENOID CAROTENOIDS
(BACTERIA)

Figure 1. Biosynthetic origins of carotenoids.

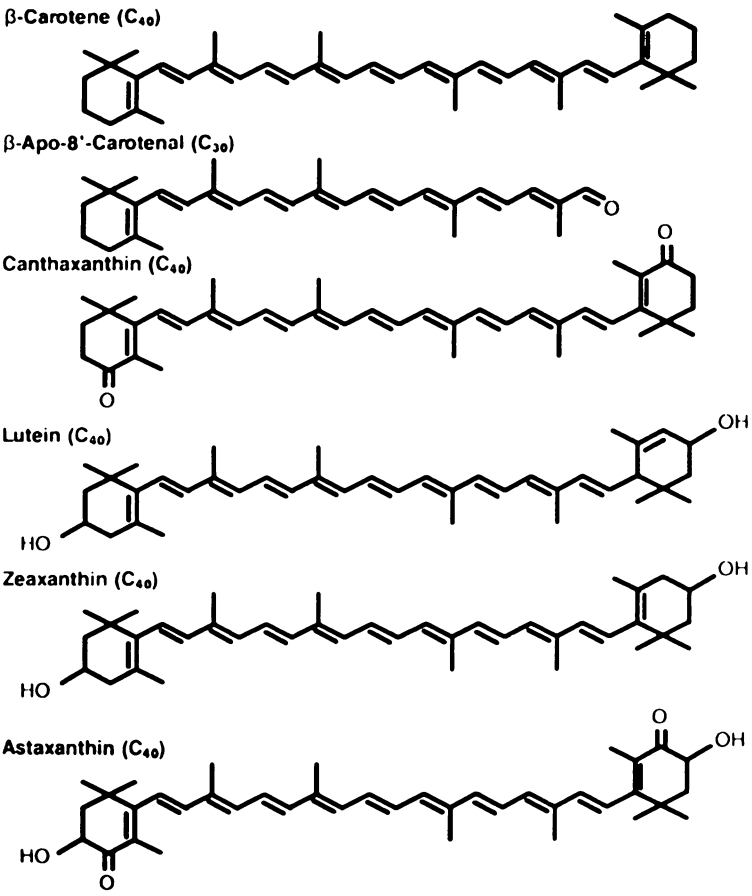
β-Carotene (C40)
β-Apo-8'-Carotenal (C30)
O
Canthaxanthin (C40)
O
O
Lutein (C40)
OH
HO
Zeaxanthin (C40)
OH
HO
Astaxanthin (C40)
O
OH
HO
O

Figure 2. Examples of commercial carotenoids.

In Regulation of Isopentenoid Metabolism; Nes, W., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1992.

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Sources of Carotenoids

Commercial carotenoids such as those shown in Figure 2 are obtained by synthesis, extraction of natural products and fermentation (Table I). Traditionally, carotenoids such as β-carotene and canthaxanthin have been produced by organic synthesis and very efficient industrial methods have been developed to produce these carotenoids in multi-ton quantities. Since extraction of natural carotenoids with organic solvents is expensive and must address problems of solvent recovery/disposal, the traditional approach in using these carotenoid-containing materials as colorants has been to use them *per se* or to use a crude extract. For example, saffron, annatto (or crude extracts) and marigold petals are used in this manner as colorants.

Table I. Sources of Commercial Carotenoids

Source	*Carotenoids (Color)*
Natural Extracts	
Annatto	Bixin, norbixin (red-orange)
Carrot	Carotenes (orange)
Palm oil	Carotenes (orange)
Saffron	Crocin, crocetin (yellow-orange)
Tomato	Lycopene (red)
Paprika	Capsanthin, capsorubin (red)
Marigold	Lutein, zeaxanthin (yellow)
Alfalfa	Carotenes (orange)
Crustacean waste	Astaxanthin (pink/red)
Fermentation	
Fungi	Carotenes (orange)
Yeasts	Astaxanthin, other xanthophylls (orange to red)
Algae	Carotenes (orange), lutein (yellow), astaxanthin (pink/red)
Chemical Synthesis	Carotenes, xanthophylls (yellow to purple-red)

This traditional approach has changed in the past decade. The potential cancer prevention functions of carotenoids has stimulated not only increased study of the pigments but also the development of new production methods for commercial carotenoids. During the past 10 years, methods have been improved or newly developed for production of carotenes and xanthophylls from natural sources. These include mass culturing of algae, fermentation and extraction from carrots and other high carotenoid content sources. Natural carotenoids such as β-carotene, lutein, astaxanthin and zeaxanthin are now commercially available.

The production of natural carotenoids can be generalized as shown in Figure 3. The natural source is harvested and then either used *per se* or extracted. Extraction can be with oils, such as corn and soybean oils, or with organic solvents such as hexane or chloroform, or by super critical fluid extraction. The oil products are sold as food colorants and vitamin supplements and typically contain from 2 to 5% carotenoid by weight. The organic solvent extracted carotenoids are purified further using fractional crystallization or column chromatography to result in crystalline solids. Carotenoids are susceptible to rapid degradation in the presence of oxygen, light and/or heat. Thus the majority of commercial carotenoids are sold as formulated products in oils which contain various stabilizers and antioxidants.

The production of natural β-carotene exemplifies the new approach being taken to natural carotenoid production. Approximately 90% of all β-carotene is currently produced by chemical synthesis such as the Roche Grignard synthesis and enol ether condensation method and the BASF ethynylation and Wittig olefin synthesis method (*17*). The production of natural carotene from sources such as carrots and microbial (fungal and yeast) fermentation was attempted by many workers but has remained difficult technically and expensive commercially. This is due to low growth yields and the expense of purifying the carotenoids.

Natural carotene production is now using more efficient culturing and harvesting methods and focussing on the development of high carotenoid-producing microbes using genetic engineering. For example, the unicellular alga *Dunalinella salina* is being mass cultured for the production of natural β-carotene by a number of commercial firms and at the Salt Research Institute (SRI), PRC (*18,19*). At the SRI, the organism is cultured in outdoor ponds up to one acre in size. After reaching a carotene content of 15 to 25g per square meter of culture, the cells are harvested by flocculation using flocculating agents such as aluminum and ferric hydroxides, ferric chloride and aluminum sulfate. The flocculated cells concentrate at the surface of the harvesting tanks and are easily skimmed off and concentrated. Acid (citric, hydrochloric or phosphoric) is added to bring the pH of the concentrated cell mass to approximately 2.5, extraction oil is added and the mass is homogenized to extract the carotene into the oil and away from the flocculent. The oil is then separated from the aqueous suspension of broken cells and flocculent by centrifugation. For a crystalline carotene product, the flocculated cells can be dehydrated by centrifugation and the cell paste is extracted with hexane or petroleum ether. The carotene extract is reduced in volume by evaporation and carotene recovered by crystallization.

In a variation on the pond method for production of β-carotene, an Australian company is harvesting lakes seeded with *D. salina* (*20*). The algae-containing brime from the lakes is passed over a hydrophobic adsorbent such as silanized glass wool, beads or diatomaceous earth (*21*). The cells adsorb to the support and, after washing, can be desorbed using less concentrated salt solutions, or can be extracted directly on the adsorbent using organic solvents.

Natural β-carotene costs from 2 to 4 times more to produce than does the synthetic compound. Yet the commercial demand for the natural product absorbs this cost differential in the quest for "natural" food ingredients. In actual fact, few

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of the natural carotene products being marketed have been fully characterized. Those that have been have been found to contain a mixture of carotenes, sterols and other lipids. For example, the extracted carotene from *D. salina* produced by the SRI shows that, in fact, the product consists of α-carotene, β-carotene and *cis*-β-carotene as shown in Figure 4 (*22*). In comparison, synthetic, crystalline β-carotene is >95% all-*trans*-β-carotene with traces of *cis*-isomers.

Biotechnology and Carotenoid Production

While some success has been achieved in the production of natural carotene, the production of natural carotenoids is still limited by low producing organisms, cultural efficiency and extraction/purification. To overcome these difficulties, a number biochemical- and biotechnology-based methods are being applied to increase carotenoid production using whole organisms and isolated carotenogenic enzymes.

Chemical Regulation of Carotenoid Biosynthesis. A number of chemicals have been found which inhibit or stimulate carotenoid biosynthesis (*23,24*). For example, diphenylamine, amitrole, acridine orange and methylene blue all inhibit carotene desaturation leading to the accumulation of lower, acyclic carotenes. Nicotine, CPTA (2-(4-chlorophenylthio)-triethylammonium chloride), triethylamine, β-ionone and many herbicides inhibit carotenoid end-group cyclization, leading to the accumulation of lycopene and other acyclic carotenes. 2,4-Dinitophenol and dimethyldioxane stimulate carotenoid synthesis in general while compounds such as 4-chlorodibenzylamine and N-benzyl phenethylamine cause the production of *cis*-carotenoids, presumably through gene derepression. Studies directed at further development of onium compounds for controlling carotenoid biosynthesis have found that, in general, compounds of the general formula $(C_2H_5)_2NCH_2CH_2R$ stimulate the production of *trans*-carotenoids while compounds having the general formula $(C_2H_5)_2NR_1R_2$ regulate the biosynthesis of *cis*-carotenoids.

Chemical regulation can provide a means to alter the production of a specific carotenoid by fermentation or in cell-free systems and has been used for this purpose.

The advent of directed mutagenesis and genetic engineering has, however, provided an alternative to the use of chemicals for increased production of specific carotenoids.

Genetic Manipulation and Engineering. The first successful attempts to genetically manipulate carotenoid production were carried out in the late 1960s (*25,26*). N-Methyl-N-nitroso-N^1-nitroguinidine induced mutants of *Phycomyces blakesleeanus* were isolated with specific and stable changes in their carotenoid biosynthetic pathway. For example, while the wild type of the fungus produces β-carotene as approximately 85% of its carotenoid content, mutant carl0(-) is blocked at carotene desaturation and accumulates nealy 100% phytoene, the colorless triene precursor of colored carotenes. Another mutant, carR21(-) is blocked at the cyclization step in carotene biosynthesis and accumulates primarily lycopene as its carotenoid end product.

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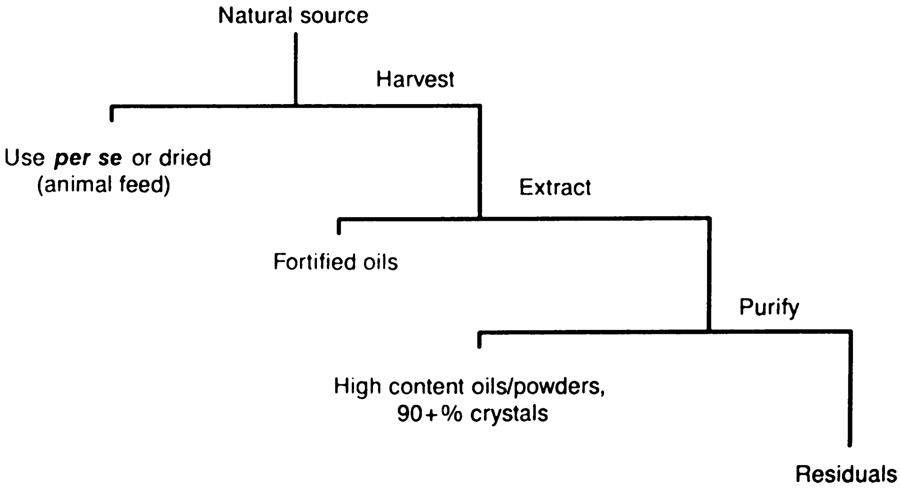
Natural source
Harvest
Use *per se* or dried (animal feed)
Extract
Fortified oils
Purify
High content oils/powders, 90+% crystals
Residuals

Figure 3. General approach for commercial production of natural carotenoids. Natural sources include bacteria, fungi, yeast, algae and higher plants.

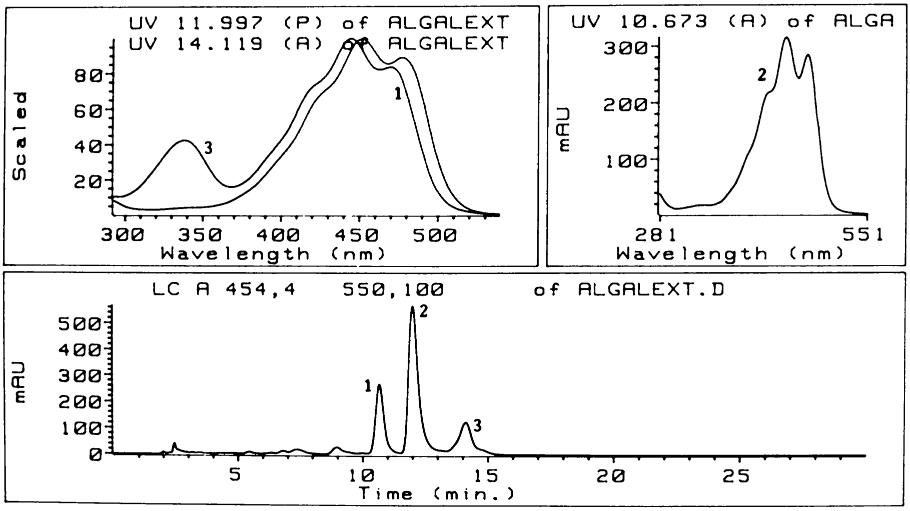

Figure 4. Diode array, reversed-phase HPLC analysis of a carotene extract from *D. salina* using a 5μM Vydac 201TP54 column (25mm x 4.6mm i.d.) and eluting with 15% acetonitrile in methanol at 1 mL/min. 1, α-carotene; 2, β-carotene; 3, *cis*-β-carotene (Adapted from ref. 22).

These first studies on carotene mutants led to new insights into carotenoid biosynthesis which, in turn, have resulted in genetic manipulation of carotenogenic encoding genes. For example, in 1986 the carotenoid gene cluster from the non-photosynthetic bacterium *Erwinia herbicola* was expressed in *E. coli* (*27*). The carotenoid containing gene cluster from photosynthetic bacteria has been mapped (*28,29*) and has been expressed in non-photosynthetic bacteria (*30*).

Other studies have focussed on expression of specific enzymes involved in carotenogenesis. For example, the *al-3* gene of *Neurospora crassa* geranylgeranyl pyrophosphate synthetase (GGPP synthase) has been cloned (*31*); and transposon mutagenesis has been used to define the carotenoid genes in *Myxococcus xanthus* and to develop non-photoinduced carotenoid producing mutants of the organism (*32*).

A number of commercial companies are also exploring genetic manipulation of carotenoid biosynthesis (*9*). For example, a research group in a major oil company has isolated the genes for GGPP synthase, phytoene synthase and phytoene dehydrogenase and expressed the genes in *E. coli*, *Agrobacterium* sp. and yeast to produce phytoene and lycopene. Two companies producing natural carotene from *D. salina* have applied strain selection and mutagenesis to produce algae producing up to 8% (dry weight) β-carotene. A high astaxanthin-producing strain of a yeast has been developed by a U.S. genetic engineering company and will be used to commercially produce this carotenoid.

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Enzyme Isolation. Currently, it is presumed that there are three to four enzymes involved in the synthesis of carotenoids to β-carotene: GGPP synthase, phytoene synthetase (phytoene synthase), phytoene desaturase and (carotene) cyclase (*24*). Some evidence suggests that GGPP synthase and phytoene synthetase activity are found on a single, monomeric protein (*33*). Isoelectric focusing analysis of extracts from carotenoid producing mutants of *P. blakesleeanus* also indicate that at least three enzymes are involved in the formation of β-carotene from GGPP (*34*).

Carotenoid biosynthetic enzymes are membrane-bound. For this reason, most biosynthetic studies on carotenoids have utilized cell-free systems (*35*). In 1985, we reported the successful solubilization of the carotenogenic enzymes from *P. blakesleeanus* using detergent extraction (*36*). Fungal mycelia were extracted with various buffered detergents and centrifuged at 105,000xg. The resulting supernatant was then assayed for incorporation of radiolabeled isopentenyl pyrophosphate (IPP) into carotenes. We found that non-ionic and zwitterionic detergents such as Tween 60 and Zwittergent 3-08 solubilized approximately 50% of the activity for conversion of phytoene to β-carotene. In addition, the presence of detergent was necessary for retention of activity during storage.

Other workers have also found that carotenogenic enzymes must be stabilized once removed from their membrane environment. For example, extraction and purification of GGPP synthase from *Capsicum* chloroplasts required chromatography and storage in buffers containing 10 to 30% glycerol (*33,38*).

More efficient separation methods are also being developed for the isolation and purification of carotenoid biosynthetic enzymes and binding proteins. For example, in 1984, we introduced the use of high performance affinity chromatography for the purification of carotenoid (and retinoid) binding proteins

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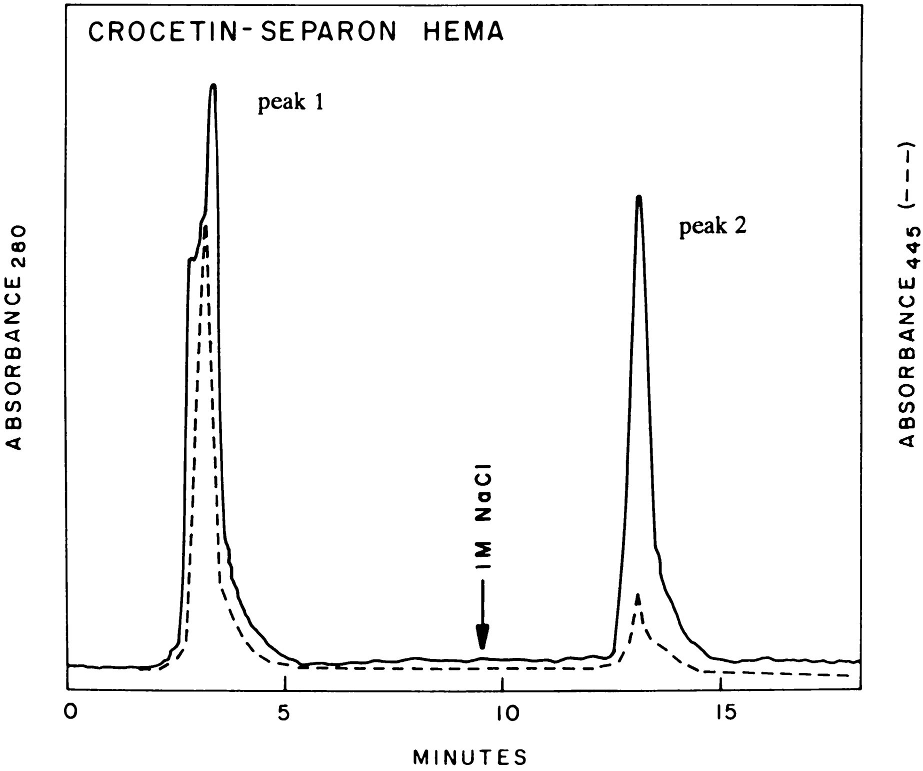

Figure 5. High performance affinity chromatography of a Triton X-100 extract of saffron on a crocetin-hyroxymeth-acrylate column (25 mm x 6mm i.d.) eluted with 0.01M Tris-HCl, pH 8 containing 1% TX-100 at 1.5 mL/min. The crocetin-binding protein (peak 2) was eluted with buffer containing 1M NaCl. The 445 nm absorbance of peak 1 is due to free crocetin in the extract.

and enzymes (*37*). Carotenoids including bixin, crocetin and torularhodin were covalently bound in dioxane or pyridine using carbodiimide or mixed anhydride coupling to a pressure stable hydroxymethacrylate support, Separon HEMA. Appropriate plant and animal extracts were then chromatographed on columns packed with the supports to isolate and purify carotenoid binding proteins. For example, Figure 5 illustrates the separation of a crocetin binding protein from extracts of saffron and *Crocus* sp. Our studies showed that affinity chromatography can result in 50 to 100X purification of a carotenoid binding protein or enzyme from a crude extract in one step in 15 to 45 min. Affinity chromatography has also been used for the purification of GGPP synthase using an aminophenyl diphosphate Sepharose support (*38*).

The isolation of carotenoid biosynthetic enzymes and/or the cloning of these enzymes opens up the potential for mass production of carotenoids using immobilized enzymes and bioreactors. A number of commercial firms are already investigating the immobilization of carotenogenic enzymes for continuous production of carotenoids. Such methods could overcome the limitations of mass culturing and provide large quantities of inexpensive natural carotenoids.

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Conclusions

The colors and unique chemistries of carotenoids have fascinated organic chemists for over 150 years, dating back to the first extraction of carotenes from carrot root by Wackenroder in 1831. Basic studies on chromatography, polyene chains and *cis-trans* isomerism by chemists such as Berzelius, Tswett, Karrer, Kuhn, Zechmeister and Heilbron utilized carotenoids as model systems. Concerted biochemical studies on carotenoids beginning in the 1950s and grounded in earlier work by scientists such as Kohl and Lederer, showed that besides having the widest distribution of any class of pigments in nature, carotenoids also appear to have the highest diversity of function: from precursor to vitamin A to phototropism and photoprotection.

Given this historical context, it is not surprising that we continue to find new functions and applications for carotenoids. The application of carotenoids as food and cosmetic colorants provided their first commercial market. The use of carotenoids in animal feeds extended this original use and now represents one of the largest markets for carotenoids. The potential use of carotenoids as anticarcinogens and radioprotective agents represent the newest and potentially their largest market.

Biotechnology is playing a central role in carotenoid chemistry and production. Genetically engineered carotenoid producing microbes and carotenogenic enzymes, immobilized enzyme systems and bioreactors, and new methods to stimulate and control carotenoid biosynthesis are actively under development. These new production methods will meet the demands for natural carotenoids as further insights are gained on their functions and applications.

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Chapter 5

Prenylated Amino Acid Composition of Tissues

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The prenylated amino acid composition of proteins has been examined by several techniques. Two of the methods, alkaline hydrolysis and hydrazinolysis, were nonspecific and indicated the presence of only farnesylcysteine and geranylgeranylcysteine. Hydrazinolysis in conjunction with mild alkaline hydrolysis indicated that a large fraction of prenylated proteins are not carboxyl methylated. The third method entailed the cleavage of prenylcysteines by 2-naphthol to yield substituted naphthopyrans, products suitable for quantitative analysis. The concentration of prenylated amino acids in mammalian tissues is 0.36 to 1.4 nmole per mg. protein, with a ratio of geranylgeranylcysteine to farnesylcysteine of about 4 to 10. The prenylcysteine content of mammalian tissue is about 1% of that of cholesterol and about equal to that of ubiquinones and dolichols. Our results indicate that about 0.5% of all proteins are prenylated.

The posttranslational modification of proteins by isoprenylation has drawn considerable attention because of the important and diverse functions of prenylated proteins. Proteins thus modified are subunits of G proteins and are implicated in regulation of cell cycle and in cancer (see *1-3* for review of this topic). Most likely, the function of prenylation is to increase the hydrophobicity of the carboxyl terminus of proteins and thus to anchor them in membranes where they function. Farnesyl and geranylgeranyl are the prenylating entities thus far identified and only cysteine at or near the carboxyl terminal region of proteins has been shown to be modified. The structure of these amino acids was established by cleavage of the thioether bond of the prenylcysteine with iodomethane (*4*) or Raney-Ni (*5,6*) and identification of the products by mass spectrometry or by comparison with standards. These structures have been verified by isolation of prenylcysteine from protein hydrolysates (*7*). Recently, fast atom bombardment mass spectrometry has been reported as an extremely sensitive method for qualitative analysis of prenylated peptides (*8*).

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Prenylated proteins may be additionally modified by methyl esterification of the prenylated carboxylterminal cysteine (*9*). This alteration has been demonstrated by the incorporation of a methyl group from radioactive methionine (*9,10*), by changes in electrophoretic mobility of the target protein (*11*), or by HPLC of proteolytic products (*12*).

While only two prenylated amino acids, farnesylcysteine and geranylgeranylcysteine, have been reported as components of proteins, we are not aware of a general search for other prenylated amino acids. Two nonspecific proteolytic procedures, alkaline hydrolysis and hydrazinolysis have been used to screen for other prenylated amino acids in metabolically labeled tissues. While geranylcysteine was found to be synthesized under the unusual conditions of mevalonate starvation no other prenylated amino acids other than farnesylcysteine and geranylgeranylcysteine were found (Rilling, H.C. *et al.*, manuscript in preparation). We have also used hydrazinolysis in conjunction with mild alkaline hydrolysis to evaluate the degree of carboxymethylation of prenylated proteins. While the identity of the prenylcysteine of many specific proteins has been established, a method for the quantitation of these two modified amino acids in tissues has been developed only recently (*13*). By this technique, we have examined a variety of tissues for prenylcysteine content.

Materials and Methods

Chemicals were reagent grade or the best available. The synthesis of prenylcysteines has been reported (*14*). CHO-K1 cells, ATCC number CCL 61, were grown and metabolically labeled as before (*15*). Three lytic procedures were used for determination of the prenylcysteine content. For total alkaline hydrolysis delipidated cellular material was suspended in 14N KOH in a Reacti-Vial (Pierce). After purging with Ar, the vial was maintained at 30° for one day. Prenyl containing proteolysis fragments were extracted into 1-butanol. Following successive washes with water, 1% aqueous trifluoroacetic acid and water, the butanol was removed under a stream of N_2 gas. Proteins also were cleaved by hydrazinolysis. Dry delipidated samples were sealed in ampules with 0.3 mL of hydrazine under Ar and heated at 100° for 12 hours, a time found to be optimal for yield of the prenylated cysteines. Hydrazine was removed *in vacuo*. Some samples were demethylated in 1N NaOH (50% ethanol-water) for 30 minutes at 25° prior to hydrazinolysis (*16*). After the addition of 1 mg of bovine serum albumin as carrier, proteins were precipitated with an equal volume of 20% concentrated HCl in methanol at 0°, washed with cold methanol, dried and subjected to hydrazinolysis. An Altex HPLC system with a Vydac C18 Protein and Peptide column was used. Farnesyl- and geranylgeranylcysteine were resolved by a gradient of 30 to 80% B over 20 minutes, where solvent A was 35% acetonitrile in water and B methanol. The solvents contained 0.1% trifluoroacetic acid and the flow was maintained at 1.5 ml/min. Authentic farnesylcysteine eluted at 15.5 minutes and geranylgeranylcysteine at 23 minutes with this program. Geranylcysteine was chromatographed isocratically, eluting at 10.5 minutes, with 26% acetonitrile in

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water as the solvent. Identity of prenylated amino acids from protein samples was verified by HPLC chromatography in a solvent system in which trifluoroacetic acid was replaced by potassium acetate buffer pH5, 10mM, in the same mixture of solvents. Synthetic geranylgeranyl- farnesyl- and geranylcysteine were used as standards and their elution position determined by absorbency at 215nM. One mL fractions were collected and radioisotope was determined by liquid scintillation spectrometry.Both procedures for proteolytic cleavage resulted in 15 to 20% recovery of protein bound radioactivity in the geranylgeranylcysteine region. The naphthol cleavage reaction was as described (*13*). Delipidated protein samples were treated with potassium 2-naphthoxide and the resulting naphthopyrans analyzed and quantitated by HPLC.

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Results

Analysis for Total Prenylcysteines and Extent of Carboxymethylation. Acid hydrolysis, a standard procedure for amino acid analysis, cannot be used for proteins bearing prenyl groups because of the sensitivity of the isoprenoid double bonds to strong mineral acids. As alternate methods for nonspecific qualitative analysis of prenyl substituted amino acids we have used alkaline hydrolysis and hydrazinolysis. Cells metabolically labeled with tritiated mevalonate were used, since all prenylated amino acids would be radioactive. Alkaline hydrolysis, under minimal conditions for hydrolysis of peptide bonds, yielded two radioactive fragments corresponding to farnesylcysteine and geranylgeranylcysteine (Figure 1). These results indicate that these are the only prenylated amino acids present. However, alkaline hydrolysis gave a substantial base line of radioactivity and we felt that other prenyl amino acids could be hidden in the chromatogram. Hydrazinolysis, another procedure for protein cleavage, was used to confirm this composition. Cleavage with hydrazine is also nonselective so that all prenyl substituted amino acids should be detected. HPLC elution profile of hydrazinolysis products from CHO proteins has a low baseline of radioactivity and shows two important products, which correspond to farnesylcysteine and geranylgeranylcysteine (Figure 2). The small satellite peaks around these amino acids, were diminished by thiolytic treatment prior to hydrazinolysis, indicating that they originated as thioethers i.e. were cysteine bound and are artifacts of hydrazinolysis.

Hydrazinolysis, as a lytic method, has a particular advantage in that it permits evaluation of the extent of carboxymethylation. If the carboxyl of an amino acid is methyl esterified or is in peptide linkage, hydrazinolysis will yield a hydrazide rather than a free carboxyl terminal cysteine. While if the prenylcysteine is free, the product is the amino acid. The extent of carboxymethylation was estimated by comparison of the hydrazinolysis products of proteins which had been demethylated by mild base treatment prior to hydrazinolysis and those that had not. The elution pattern of the hydrazinolysis products from alkaline treated CHO cells is shown by the closed circles in Figure 2. If the hydrolysis was omitted, there was a substantial decrease in the amount of farnesylcysteine found, while there was little change in geranylgeranylcysteine (Figure 2, closed circles). This relative change was confirmed in a duplicate experiment, in which demethylation led to a 2.8 fold

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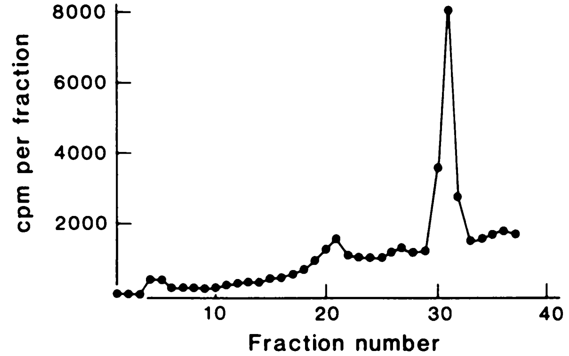

Figure 1. HPLC of strong alkaline hydrolysates of CHO proteins.
The conditions for chromatography are given in methods. Farnesylcysteine elutes in fractions 21 and 22 and geranylgeranylcysteine in fractions 32 and 33.

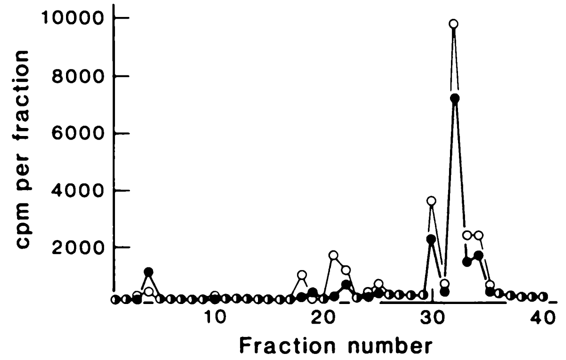

Figure 2. HPLC of the hydrazinolysis products from CHO proteins obtained from cells labeled in media containing 6 μM mevalonate. Conditions for labeling as well as chromatography are the same as are indicated in figure 1. The open circles are the patterns obtained from samples treated with mild base before hydrazinolysis. The closed circles are from a sample that was not subjected to mild alkaline hydrolysis.

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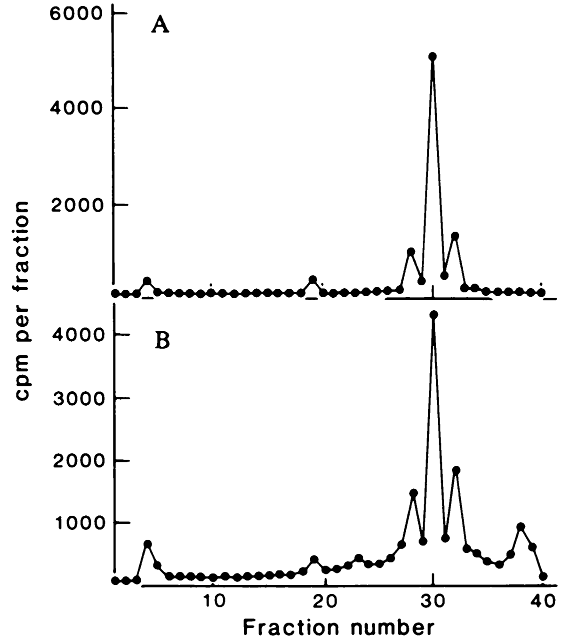

Figure 3. HPLC of the hydrazinolysis products obtained from mouse kidney proteins with (panel A) and without (panel B) demethylation by mild alkaline treatment. The conditions of chromatography are the same as for figure 2.

increase in farnesylcysteine recovery with a concomitant 50% increase in geranylgeranylcysteine. This indicates that a significant fraction of farnesylcysteine is carboxymethylated, while substantially less geranylgeranylcysteine is so modified. The apparent low level of carboxymethylation led us to examine another readily labeled tissue, mouse kidney (*17*), by the same procedure. The data, figure 3, shows that there is also relatively little carboxymethylation of prenylcysteines in this tissue.

While these procedures for proteolysis gave a satisfactory qualitative analysis of the prenyl amino acid composition of proteins, neither was satisfactory for quantitation. We, therefore, developed a method for analysis that fortuitously took advantage of a unique reaction of the allylic thioether bond of prenylcysteines.

Our initial reasoning was based upon the possibility of the allylic prenyl group being displaced from the cysteinyl derivative by a suitable nucleophile which contained a strongly absorbing chromophore. A number of displacing agents were considered and tested but the naphthoxide ion appeared to be the most effective. Although the expected products, naphthyl prenyl ethers, were not observed, stable cyclic derivatives with a modified naphthalene ultraviolet absorption spectra were formed (18,19). These products were quite suitable for quantitative studies. The elution profile of the naphthol cleavage products from mouse kidney are shown in figure 4.

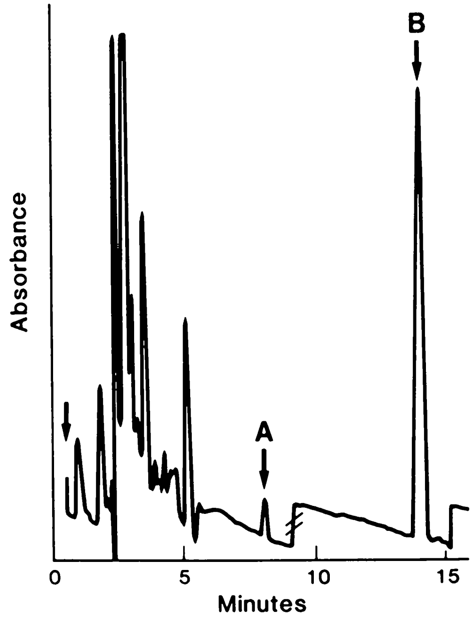

Figure 4. HPLC of naphthol cleavage products from mouse kidney. The column was eluted with a gradient of 95% acetonitrile/water to acetonitrile over 15 min. Detection was at 360 nm and the arrow indicates sample injection. A is farnesyl cysteine and B geranylgeranyl cysteine.

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Analysis of Tissues by Naphthol Cleavage. The naphthol dependent cleavage of prenylcysteine permits analysis of unlabeled tissues with a sensitivity of 1 pmole prenylnaphthopyran and a recovery of 22% from the prenylcysteines of protein (*13*). A variety of samples, intended to cover a wide range of organisms as well as individual tissues, were screened for prenylcysteine content by this reaction. Cultured mammalian cells (CHO, 3T3, a neuroblastoma and a Murine fibrosarcoma) were found to have about 0.7 nmole prenylcysteine per mg of protein with a ratio of geranylgeranyl- to farnesylcysteine of about 6 (*13*). Various tissues from mouse had a similar composition but with a greater range in the ratio of farnesylcysteine to geranylgeranylcysteine (Table I). Not all prenylated proteins were membrane bound, since sedimentation of membranes at high speed removed only about 60% of prenylated proteins.

The results from analyses of non-mammalian tissues are shown in Table II. The prenyl content is significantly lower and the fraction of the sesquiterpene amino acid is greater than in mammalian cells. Prenylcysteines were not found in *E. coli*.

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Table I. Prenylcysteine composition of mouse tissues

Tissue	Prenylcysteine nmole/mg. protein	GGcys/Fcys*
Liver	.32	6
supernatant	.15	4
Kidney	.75	8
supernatant	.23	7
Brain	1.3	10
Lung	.64	5

SOURCE: Reprinted from ref. 13.

* The ratio of geranylgeranylcysteine to farnesylcysteine.

Table II. Prenylcysteine composition of other organisms

Organism	Prenylcysteine nmole/mg. protein	GGcys/Fcys
E. coli	nil	
Methanobacterium thermoautotrophicum	.04	*
S. cerevisiae	.05	3
" " a factor strain	.05	.7
Phycomyces	.22	3
Peletia fastigiata brown algae	.06	†
Spinacia oleracea	.06	†
Manduca sexta		
larvae	.14	3
eggs	.14	4

SOURCE: Reprinted from ref. 13.

* This sample had no detectable geranylgeranylcysteine.

† These samples had only traces of farnesylcysteine.

Discussion

We have analyzed proteins from several tissues for prenyl amino acids by three different procedures. The two proteolytic procedures used, hydrazinolysis and alkaline hydrolysis, should yield all prenylated amino acids regardless of structure and with these methods we found only geranyl-farnesyl- and geranylgeranylcysteine (Figures 1-3). For the present, geranyl cysteine found under certain circumstances must be considered an artifact of the conditions that we used for incorporation of radioactive mevalonate. Cells that elaborated geranylcysteine were starved for mevalonate by treatment with lovastatin and then fed minimal amounts of the isoprenoid precursor. Hydrazinolysis, in conjunction with demethylation, has also been used to estimate the extent of methylation of carboxyl terminal prenylcysteines. The recovery of farnesylcysteine was considerable less from protein samples that had not been demethylated while there was only a small decrease in geranylgeranylcysteine under these conditions (Figures 2 and 3). These results demonstrate that most of the farnesylcysteine in proteins is carboxymethylated while substantially less geranylgeranylcysteine is thus modified. When kidney proteins were examined by the same procedure, they were found to be sparingly methylated. Because of low yields of hydrazinolysis products and the lack of an internal standard, these results are only qualitative. None-the-less they indicate that terminal methylation is not general.

In searching for a method for quantifying prenylcysteines we found, to our surprise, that naphthopyran derivatives 1 and 2 (Scheme 1) were formed when protein was allowed to react with potassium naphthoxide. Structures for these products were established by model studies as well as by synthesis of 1 and 2 for comparison (Epstein, W.W. *et al.*, manuscript in preparation, 18,19). A rationalization for formation of the naphthopyrans involves the known ambident nature of the naphthoxide ion. Allylation of the naphthalene nucleus with the prenyl part of the prenylated protein could occur as shown in Scheme 1.

The allylated product would be in equilibrium with its enol form and cyclization of these derivatives upon heating is known (*18,20*) requiring only dehydrogenation to form 1 and 2.

The nucleophilic cleavage of the allylic thiol bond by 2-naphthol provides for qualitative as well as semi-quantitative determination of the cysteine bound prenyl groups of prenylated proteins. The method has several advantages. The naphthopryans that are formed are readily isolated and quantified by HPLC. Also, the tissue to be analyzed does not need to be metabolically labeled with mevalonate. The extinction coefficients of the model naphthopryan are 4450 and 66070 L/mole and at 360 and 244 nm respectively[2]. At 246 nm the maximum sensitivity is about 1 pmoles of naphthopryan.

Naphthol cleavage of prenylcysteines has enabled us to examine different tissues for the presence of this modification and to extend the range of organisms in which it is known to occur (Tables I and II). Our results clearly indicate that this modification of proteins is very nearly universal. The level of modified cysteine is lower in plants and insects than in mammals and lower yet in an archaebacterium. Prenylcysteines were not found in *E. coli* at a sensitivity that would have detected

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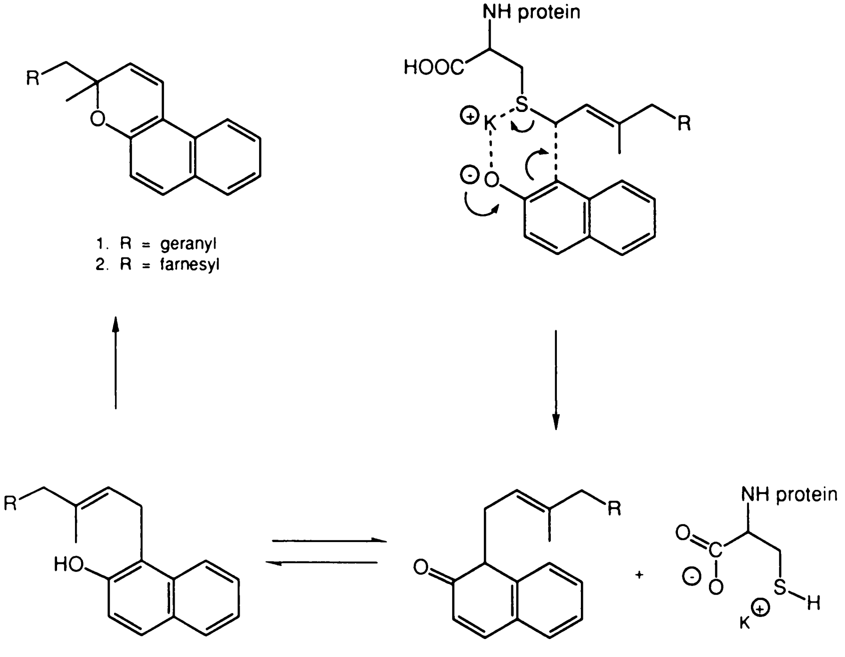

Scheme 1

5 pmole prenyl residue per mg protein. Thus, proteins so modified may not occur in all organisms. The tissue distribution of prenylated proteins parallels the enzymatic activity reported for protein farnesyltransferase as reported by Manne *et al.* (11). They detected this enzyme in extracts of several mammalian tissue but failed to find activity in extracts of *E. coli.*

While the function of prenylation is to direct selected proteins to membrane compartments it is apparent that this modification is not sufficient for quantitative transfer, since we found that one third to one half of these proteins are in the cytosolic pool. This observation in agreement with that of Maltese and Sheridan (*12*) who detected significant levels of cytosolic prenylated proteins. Methylation, after prenylation, is also an important factor for partitioning of prenylated proteins into membranes. Hancock, Cadwallader and Marshall measured the membrane binding of farnesylated ras proteins as a function of carboxymethylation and determined that methylation inceased membrane binding from 40 to about 70% (21). This finding serves to emphasize our observation that methylation of these proteins is far from quantitative.

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Chapter 6

Isoprenylation of Low Molecular Mass Guanine Nucleotide-Binding Proteins in Mammalian Cells

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Specific groups of proteins in mammalian cells are modified post-translationally by farnesyl or geranylgeranyl isoprenoids which are linked via thioether bonds to cysteines within defined carboxyl-terminal amino acid motifs. In the case of $p21^{ras}$, the minimal protein sequence recognized by the farnesyltransferase consists of four carboxyl-terminal residues; C-*a*-*a*-S or C-*a*-*a*-M, where the *a*'s represent aliphatic amino acids. Other low molecular mass GTP-binding proteins (*e.g.*, G25K, *rap*1A, *rap*1B, *rho*A, *rac*1 and *rac*2) are modified by geranylgeranyl groups. These proteins are distinguished from the *ras* proteins by the presence of L instead of M or S in the terminal position of the C-*a*-*a*-*x* sequence. Changing the terminal residue in $p21^{ras}$ from S to L by site-directed mutagenesis results in the protein being modified by geranylgeranyl instead of farnesyl, implying that substrate specificity for farnesyltransferase or geranygeranyltransferase is determined by the last amino acid in the C-*a*-*a*-*x* motif. Proteins of the *rab* family contain carboxyl-terminal sequences that differ substantially from the *ras*-like C-*a*-*a*-*x* motif; i.e., *x*-C-*x*-C (*rab*3, *rab*4, *rab*6), and G-G-C-C (*rab*1, *rab*2). These proteins are modified by geranylgeranyl groups *in vitro* and *in vivo*. The *rab*1B protein is a poor substrate for isoprenylation when either C is removed or when either G is changed to a D in the C-terminal G-G-C-C sequence. Two distinct protein:prenyltransferases have been isolated from mammalian tissues; one transfers farnesyl to proteins with the C-*a*-*a*-M/S motif and the other transfers geranylgeranyl to proteins with the C-*a*-*a*-L terminus. However, based on the diversity of carboxyl-terminal sequence motifs now known to undergo cysteine modification by isoprenoids, it is probable that additional protein:prenyltransferases will be identified, each having specificity toward a discrete class of target proteins.

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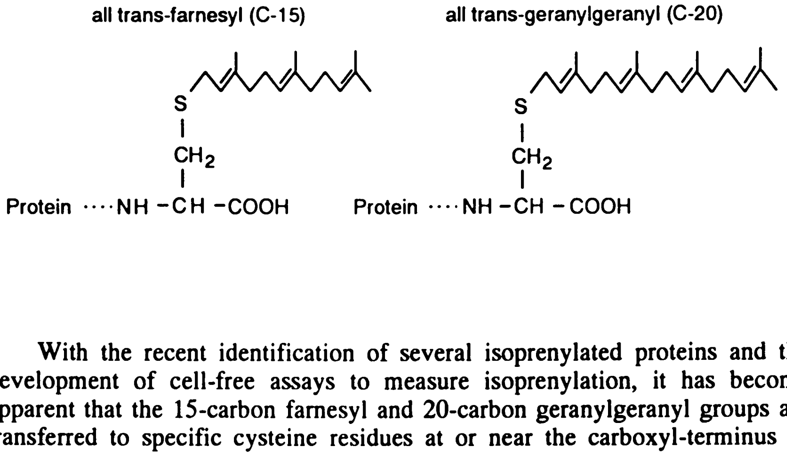
In 1984 Schmidt *et al.* (*1*), noted that cultured mouse fibroblasts were able to incorporate isoprenoids derived from radiolabeled mevalonate into proteins. Although covalent modification of peptides by farnesol and hydroxyfarnesol had been described earlier in fungal mating factors (*2-4*), this constituted the first evidence for isoprenylation of proteins in mammalian cells. Isoprenylation is an immediate post-translational modification. That is, the isoprenyl moiety appears to be transferred to the protein very soon after synthesis of the polypeptide chain is completed on the ribosomes (*5*). As in the fungal mating peptides, the isoprenyl groups associated with mammalian proteins are linked to cysteine residues via thioether bonds. Hence, they can be released from the proteins as alcohols by reaction with methyl iodide and cleavage of the sulfonium salt (*2,6-8*) or as isoprenyl hydrocarbons by reaction with Raney nickel catalyst (*9-12*). Structural analysis by gas chromatography and mass spectroscopy has revealed the existence of two types of modifying isoprenyl groups in mammalian proteins; all-*trans* farnesyl and all-*trans* geranylgeranyl (*9-16*).


all trans-farnesyl (C-15)
all trans-geranylgeranyl (C-20)

With the recent identification of several isoprenylated proteins and the development of cell-free assays to measure isoprenylation, it has become apparent that the 15-carbon farnesyl and 20-carbon geranylgeranyl groups are transferred to specific cysteine residues at or near the carboxyl-terminus of the acceptor protein by enzymes that utilize the corresponding farnesyl or geranylgeranyl pyrophosphates as substrates (reviewed in refs. *17-19*).

We are studying a family of mammalian proteins which are known to bind and hydrolyze guanine nucleotides (*i.e.*, the low molecular mass GTP-binding proteins), with the aim of applying molecular cloning techniques and site-directed mutagenesis to identify the structural signals that permit proteins to be recognized by the isoprenyltransferase enzymes. This paper will summarize the current understanding of the specificity of protein isoprenylation that has emerged from these studies.

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Differential Distribution of Farnesyl and Geranylgeranyl in Cellular Proteins

Mevalonate serves as a precursor for all cellular isoprenoids, including sterols, dolichols and the polyprenyl side chains of ubiquinones (for review see ref. *20*). When cultured cells are incubated in medium containing [^{3}H]mevalonate, relatively little radioactivity is incorporated into polypeptides, presumably because the radiolabeled mevalonate taken up by the cells is diluted by the endogenous mevalonate pool. However, when cells are incubated concurrently with [^{3}H]mevalonate and a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (*e.g.*, compactin or mevinolin/lovastatin), the endogenous synthesis of mevalonate is blocked and incorporation of radiolabeled mevalonate-derived isoprenoids into proteins is easily detected.

The [^{3}H]mevalonate-labeled proteins from many mammalian cell lines display a complex but fairly reproducible profile when resolved according to relative molecular mass by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by fluorography (for example, see Figure 1, lane 1). Invariably, the most heavily labeled group of proteins is found between 20 kDa and 30 kDa, but radiolabled proteins are also clearly visible at 17 kDa, 45 kDa, 53 kDa and 66-70 kDa. The pattern of isoprenylated proteins becomes even more complex when the proteins are separated on two-dimensional gels, using isoelectric focusing in the first dimension and SDS-PAGE in the second dimension (*18,21,22*).

The reported existence of two types of protein-bound isoprenyl groups within a single cell type (*10*) initially raised two alternative possibilities regarding the specificity of protein prenylation: 1) The chain-length of the isoprenyl group attached to a particular protein is dependent on the relative abundance of farnesyl pyrophosphate *versus* geranylgeranyl pyrophosphate within the cell and is not protein-specific, or 2) Proteins contain specific amino acid sequence elements that serve as recognition signals for different prenyltransferases, so that individual proteins act as acceptors for either farnesyl or geranylgeranyl groups. Studies combining pharmacologic manipulation of cultured cells with *in vitro* assays for protein prenylation have provided clear evidence to support the latter possibility.

Early in our studies of protein isoprenylation, we noted that cells treated with lovastatin rapidly exhausted their endogenous supply of isoprenyl pyrophosphates and began to accumulate pools of non-isoprenylated substrate proteins (*5*). In most cases, these non-isoprenylated proteins appear to accumulate in the cytosolic compartment of the cell (*22,23*). Thus, we reasoned that if lovastatin-treated cells were disrupted in a buffer suitable for preserving protein:prenyltransferase activity, and the soluble fractions were incubated with either [^{3}H]geranylgeranyl pyrophosphate or [^{3}H]farnesyl pyrophosphate, it might be possible to determine whether modification of the accumulated substrate proteins was selective or non-selective. The results of such an experiment are depicted in Figure 1 (lanes 2 & 3). It can be seen that while some of the proteins in the cell lysate were labeled only with farnesyl, others were labeled predominantly with geranylgeranyl, supporting

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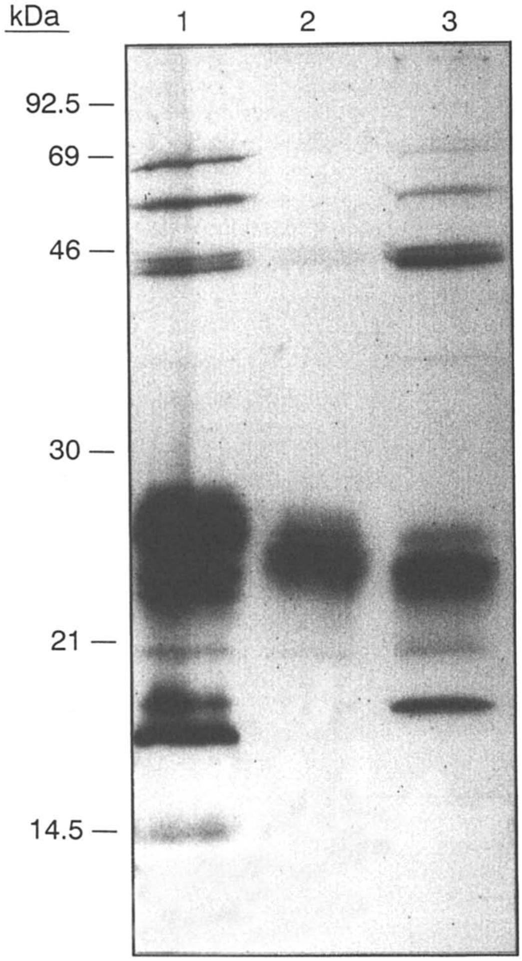

Figure 1. Electrophoretic profiles of isoprenylated proteins in cultured mouse erythroleukemia cells. Lane 1: Protein-bound isoprenyl groups were metabolically labeled by incubating cells in medium containing [^{3}H]mevalonate and 25 µM lovastatin. Total cellular proteins were then separated by SDS-PAGE and the radiolabeled proteins in the gel were visualized by fluorography (see refs. 5 & 22 for detailed methods). Positions of standard proteins of known molecular mass are indicated at the edge of the panel. Lanes 2 & 3: Cells were pre-incubated for 18 h with 25µM lovastatin, then lysed in an equal volume (50 µl) of a solution consisting of 50 mM Tris-HCl, pH 7.0, 10 mM dithiothreitol, 10 mM $MgCl_2$. The supernatant solution remaining after centrifugation at 100,000 x g for 30 min was then incubated for 1 h at 37°C with 1 µCi (2.5 µM) [^{3}H]geranylgeranyl pyrophosphate (lane 2) or 1 µCi (2.5 µM) [^{3}H]farnesyl pyrophosphate (lane 3). The radiolabeled proteins were resolved by SDS-PAGE in parallel with the proteins from the cells labeled *in vivo* with [^{3}H]mevalonate (lane 1).

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the notion that different sets of proteins are recognized specifically by different prenyltransferase enzymes. However, in examining the electrophoretic profiles of proteins labeled *in vitro* with [^{3}H]geranylgeranyl or [^{3}H]farnesyl (Figure 1, lanes 2 & 3), it is evident that some of the proteins that are metabolically labeled by [^{3}H]mevalonate *in vivo* (Figure 1, lane 1) are not detected in the soluble cell lysates. We have confirmed that additional proteins in the particulate fraction are not labeled with radiolabeled isoprenyl groups *in vitro* (Maltese & Sheridan, unpublished). Therefore, it is probable that this kind of assay does not demonstrate all of the potential substrate proteins and isoprenyltransferase activities that are normally represented in the intact cell.

As an alternative to the assay described above, we devised a simple approach for estimating the chain-lengths of radiolabeled isoprenyl moieties associated with discrete groups of proteins in cultured cells incubated with [^{3}H]mevalonate (*24-26*). Briefly, the proteins are resolved by SDS gel electrophoresis and the [^{3}H]mevalonate-labeled proteins in defined regions of the gel are isolated by electroelution. After incubation with Raney-nickel to cleave the thioether bonds (*10,11*), the radiolabeled isoprenyl groups released from a given protein or set of proteins are extracted into pentane, reduced over platinum oxide (*10,11*) and subjected to high performance gel permeation chromatography along with various hydrocarbon standards. The standards are detected by monitoring the refractive index of the column effluent, while elution of the radiolabeled hydrocarbons derived from the proteins is monitored by liquid scintillation counting (*24-26*). An example of this type of analysis, applied to total proteins from simian COS cells, is shown in Figure 2. The results again indicate that 15-carbon and 20-carbon isoprenyl chains are associated with discrete proteins. Similar results were obtained with other mammalian cell lines (*24*), supporting the notion that different sets of target proteins are recognized by at least two isoprenylating enzymes with unique specificties for farnesyl or geranylgeranyl pyrophosphate substrates.

The C-*a*-*a*-*x* Motif as a Structural Signal for Protein Isoprenylation.

Recent progress in defining the structural signals that mark proteins as targets for isoprenylation stems largely from the recognition of similarities between the farnesylated fungal mating factors and mammalian cell proteins. For example, studies of the mature *Saccharomyces cerevisiae* **a**-mating factor indicated that the farnesylated cysteine is located at the carboxyl-terminus of the peptide and is methylated (*4*), whereas the gene coding for the mating factor predicts a peptide with an extension of three amino acids beyond the target cysteine (--C-V-I-A) (*27*). Thus, it was discovered that the farnesylation of cysteine in the fourth position from the carboxyl-terminus initiates a series of post-translational processing events that includes proteolytic removal of the three terminal amino acids and methylation of the exposed carboxyl group of the farnesylated cysteine. The first two classes of isoprenylated proteins identified in mammalian cells were pre-lamin A and lamin B (*28-30*), structural proteins of the nuclear matrix, and the *ras* GTP-

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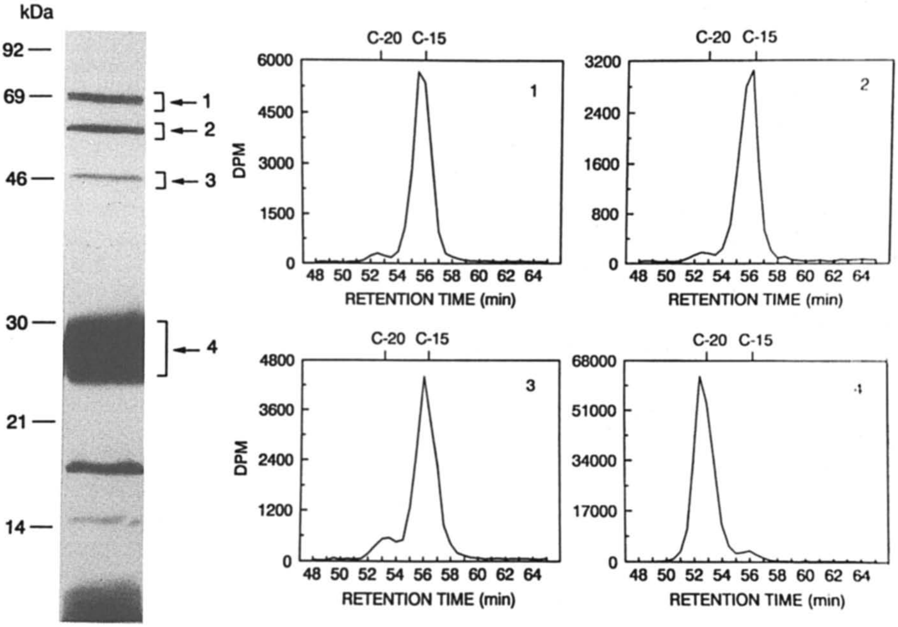

Figure 2. Determination of the chain-lengths of radiolabeled isoprenyl groups associated with proteins in cultured COS cells. Cells were incubated for 18 h with [^{3}H]mevalonate. Isoprenylated proteins were separated by SDS-PAGE and visualized by fluorography (positions of the molecular mass standards are indicated on the left). Specific zones indicated by the numbered arrows were excised from adjacent sections of the non-fluorographed gel, and the proteins were electroeluted. Radiolabeled isoprenyl groups were released from the proteins by Raney-nickel cleavage, hydrogenated over platinum, and characterized by gel permeation chromatography (see ref. 24 for details). The chromatograms of the radiolabeled hydrocarbons derived from the isoprenylated proteins are numbered to correspond to the zones of the gel from which they were eluted. The retention times of the 15-carbon (farnesane) and 20-carbon (phytane) standards are shown at the top of each chromatogram. (Reproduced with permission from ref. 24. Copyright 1991 Kluwer Academic Press)

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binding proteins (*7,23,31*), implicated in growth regulation and signal transduction (*32,33*). In each case the modifying group appeared to be a farnesyl moiety, and the predicted protein sequence ended with a C-*a*-*a*-*x* motif (where the *a*'s are amino acids with aliphatic or hydroxyl side chains and the *x* is any amino acid). In the case of $p21^{ras}$, the farnesylation was required for subsequent proteolytic processing and carboxylmethylation steps, similar to those described in the fungal mating peptides (*7,23,31,34-37*). In light of these findings, it was postulated that the C-*a*-*a*-*x* sequence might constitute a general structural feature by which proteins are recognized as substrates for isoprenylation. Evidence to support this hypothesis has come from subsequent reports describing isoprenylation of a number of proteins with C-*a*-*a*-*x* motifs at their carboxyl-termini. These proteins include the γ subunits of retinal transducin (*13,14*) and heterotrimeric brain G-proteins (*8,12,38,39*), as well as several *ras*-related low molecular mass GTP-binding proteins, such as *rap*1A (*40*), *rap*1B (*15*), *rap*2 (*41*), *rho*A (*42*), G25K (*16,22*), *rac*1, *rac*2 and *ral*A (*26*). However, not all proteins with carboxyl-terminal sequences fitting the C-*a*-*a*-*x* pattern undergo isoprenylation, as indicated by the failure to detect $[^3H]$mevalonate incorporation into the α subunits of the heterotrimeric G-proteins (carboxyl terminal sequences: C-G-L-F, C-G-L-Y) (*38,39,43*). Interestingly, while the modifying group attached to the transducin γ subunit was the same as that found in $p21^{ras}$ and lamin B (*i.e.*, farnesyl), the other *ras*-related GTP-binding proteins cited above were modified by 20-carbon isoprenoids. Thus, attention has now focused on resolving three major issues: 1) What is the minimal structural unit required for protein recognition by the appropriate isoprenyltransferases? 2) What combinations of adjacent carboxyl-terminal amino acids will permit isoprenylation of the cysteine? 3) What are the structural signals that mark proteins as targets for modification by 15-carbon farnesyl or 20-carbon geranylgeranyl. Recent studies employing molecular biological approaches as well as classic protein purification and enzymology have shed considerable light on these issues.

Manipulation of the C-*a*-*a*-*x* Sequence by Site-Directed Mutagenesis.

Studies conducted by Hancock et al. (*23*) initially demonstrated that by cloning the sequence coding for the last four amino acids (C-V-L-S) of $p21^{H\text{-}ras}$ into a vector containing the sequence coding for a heterologous protein (protein A), a hybrid protein was obtained (protein A--C-V-L-S) that was capable of undergoing metabolic labeling with $[^3H]$mevalonate. This indicated that the $p21^{ras}$ C-*a*-*a*-*x* sequence was sufficient to mark the protein as a substrate for isoprenylation. Further manipulation of the *ras* C-*a*-*a*-*x* motif by site-directed mutagenesis has revealed that the -*a*-*a*-*x* extension plays an important role in isoprenyltransferase recognition. For example, when a truncated K-*ras*B protein was produced by inserting a stop codon after the cysteine codon, the protein ending with cysteine was unable to undergo isoprenylation (*36*). The length of the amino acid extension following the cysteine is also critical, since shortening the *ras* C-V-L-S sequence to C-V-

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L (*44*) or C-T-P (*45*) or lengthening it to C-V-L-S-A (*44*) renders the protein a poor substrate for farnesylation.

TABLE I. Effects of carboxyl-terminal alterations on isoprenylation of *rac*1, *rac*2 and *ral*A-proteins

	Amino Acid Sequence[a]	*Relative* $[^3H]$*Mevalonate Incorporation*[b]
	*rac*1	
(wt)	K-R-K-C-L-L-L *	++
	K-R-K *	--
	K-R-K-W-L-L-L *	--
	K-R-K-C-C-C-C *	--
	K-R-K-C-R-L-L *	++
	*rac*2	
(wt)	K-R-A-C-S-L-L *	++
	K-R-A *	--
	K-R-A-W-S-L-L *	--
	K-R-A-C *	--
	ralA	
(wt)	R-E-R-C-C-I-L *	++
	R-E-R-C-S-I-L *	++
	R-E-R-S-C-I-L *	--

* Represents carboxyl terminus.
[a] Changes introduced by site-directed mutagenesis are underlined.
[b] See ref. *26* for actual fluorograms and incorporation values.
(Adapted from ref. *26* with permission, copyright 1991)

We have explored the structural requirement for geranylgeranyl modification by creating various mutations in the genes coding for *rac*1, *rac*2, and *ral*A and translating the altered mRNAs in rabbit reticulocyte lysates containing $[^3H]$mevalonate (*26*). Since enzymes in the reticulocyte lysates can convert mevalonate to isoprenyl pyrophosphates and transfer both farnesyl and geranylgeranyl groups to proteins, this approach permits a detailed analysis of the consequences of carboxyl-terminal sequence alterations on the ability of proteins to serve as isoprenyl acceptors (see Table I).

As in the case of p21^{ras}, the *rac* proteins engineered to end with cysteine were not good substrates for isoprenylation, and placement of cysteine in the third position from the carboxyl terminus in the *ral*A protein abolished the modification. These studies also suggested that the amino acid occupying the third position in the C-*a*-*a*-*x* motif need not have an aliphatic side chain, since proteins with serine, cysteine or arginine in this position were able to undergo isoprenylation *in vitro*.

In considering the possible structural basis for the modification of many low molecular mass GTP-binding proteins by geranylgeranyl rather than farnesyl, we noted that in every case where a 20-carbon modification was confirmed (*8,12,15,16,26,40,42*), the terminal *x* position in the C-*a*-*a*-*x* motif

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was occupied by leucine. In contrast, the known farnesylated proteins and peptides end with methionine, serine or alanine (*4,7,11,13,14*). To assess the potential significance of this difference in the *x* position, we changed the terminal amino acid in p21^{Hras} (C-V-L-S) from serine to leucine by site-directed mutagenesis. The mutant protein (C-V-L-L) then behaved as a specific acceptor for [^{3}H]geranylgeranyl rather than [^{3}H]farnesyl *in vitro* (*46*). Futhermore, when the altered H-*ras* gene was transfected into COS cells and the overexpressed protein was metabolically labeled with [^{3}H]mevalonate, the radiolabeled isoprenyl moiety removed from the immunoprecipitated *ras* protein behaved as a 20-carbon chain when subjected to gel permeation chromatography, confirming that the altered substrate specificity of the protein was maintained *in vivo* (*46*). These studies suggest that in proteins ending with a C-*a*-*a*-*x* motif, recognition by farnesyltransferase or geranylgeranyl transferase is specified by the amino acid occupying the terminal position, with leucine representing a signal for geranylgeranylation.

Studies with Purified Protein:Isoprenyltransferases.

A mammalian p21^{ras}:farnesyltransferase has recently been purified to homogeneity from rat brain (*47*). The enzyme is a heterodimer consisting of α and β subunits of 49 kDa and 46 kDa respectively. Cross-linking studies indicate that the β subunit binds p21^{ras} (*48*). The availability of purified enzyme has afforded new oportunities for defining the sequence requirements for protein recognition by farnesyltransferase. Reiss *et al.* (*49*) tested an extensive series of synthetic tetrapeptides modeled after the carboxyl termini of known proteins for their ability to compete with recombinant p21^{Hras} as substrates for farnesylation. Peptides based on the natural substrates for farnesylation (*i.e.,* those ending with methionine or serine) were good competitors, whereas those based on known geranylgeranylated proteins (*i.e.,* those ending with leucine) were poor competitors. These studies also provided information about the relative importance of the two aliphatic amino acids in the second and third positions from the carboxyl terminus. Thus, in agreement with our work involving site-directed mutants of *rac*, some nonaliphatic substitutions were permitted in the position immediately distal to the cysteine. However, substitutions of nonaliphatic residues in the second position from the carboxyl terminus generally reduced the ability of the peptides to compete against the farnesyltransferase (*49*).

The geranylgeranyltransferase involved in modifying proteins with a C-*a*-*a*-*x* carboxyl terminus has not yet been purified to homogeneity, but several partial purifications have been reported (*50-52*). The geranylgeranyl transferase appears to be a distinct enzyme, based on it separation from the protein:farnesyltransferase by ion-exchange chromatography (*50-52*). However, immunoprecipitation studies indicate that the enzyme is a heterodimer sharing the same α subunit as the farnesyltransferase (*51*). This implies that the recognition of specific protein substrates is a function of the β subunit. As predicted, proteins with C-*a*-*a*-*x* motifs ending with leucine were the best substrates for the geranylgeranyltransferase (*50-52*). However, it is noteworthy that at high concentrations of acceptor peptide, the partially purified geranylgeranyltransferase exhibits relaxed substrate specificity and can transfer either geranylgeranyl or farnesyl to some peptides (*52*). It remains to be seen whether proteins that normally undergo geranylgeranyl modification *in vivo* can be modified by farnesyl under specific physiological conditions.

Isoprenylation of Cysteines in Proteins without a Classic Carboxyl-Terminal C-*a*-*a*-*x* Motif

In mammalian cells the *rab* genes encode a set of 22-26 kDa GTP-binding proteins that are believed to play a role in facilitating the vectorial movement of exocytic and endocytic vesicles (for review see ref. *53*). Similar proteins, YPT1p and SEC4p, are required for specific steps in the yeast secretory pathway (*54,55*). Although all of the *rab* proteins have cysteine residues within the last four amino acids of their carboxyl-terminal sequences, the positioning of these cysteines is different from the C-*a*-*a*-*x* pattern of p21^{ras} and other *ras*-related proteins mentioned earlier. For instance, the *rab*1 and *rab*2 proteins end with a *x*-*x*-C-C motif, and the *rab*3, *rab*4 and *rab*6 proteins end with *x*-C-*x*-C (*56*). Prompted by studies indicating that the carboxyl-terminal regions of the YPT1 and SEC4 proteins are essential for their interactions with cellular membranes (*54,55*), we sought to determine whether the related *rab* proteins might undergo isoprenylation. Using an *in vitro* assay in which various *rab* cDNA's were transcribed and their RNA's were translated in reticulocyte lysates containing [^{3}H]mevalonate, we determined that the *rab*1B, *rab*2, *rab*4, *rab*5 and *rab*6 proteins were capable of serving as isoprenoid acceptors (Figure 3). Gel permeation chromatography of the radiolabeled hydrocarbons released from the proteins indicated that the mevalonate-derived isoprenyl groups were of the 20-carbon class (*25*). More recently, we found that the same proteins act as specific acceptors for [^{3}H]geranylgeranyl, but not [^{3}H]farnesyl, *in vitro* (Figure 3). Analyses of various *rab* proteins expressed in mammalian (COS) cells (*57*) or insect (Sf9) cells (*58*) have confirmed that they are modified by 20-carbon isoprenyl derivatives of [^{3}H]mevalonate *in vivo*.

The modification of *rab* proteins terminating with cysteine was surprising in light of the earlier work with *ras* and *rac* proteins (*26,36*) which indicated that truncated proteins without the *a*-*a*-*x* portion of the C-*a*-*a*-*x* motif were poor substrates for isoprenylation. The results suggested that isoprenyltransferases distinct for those involved in modifying C-*a*-*a*-*x* proteins might be responsible for modifying the proteins of the *rab* family. To further define the carboxyl-terminal features essential for isoprenylation of proteins ending with the *x*-*x*-C-C motif, we constructed several variants of the *rab*1B cDNA by site-direceted mutagenesis and assessed the ability of the altered proteins to serve as substrates for isoprenylation in the *in vitro* translation/isoprenylation asssay (*25*). The results, which are summarized in Table II, indicate that both of the terminal cysteines must be present for optimal isoprenylation. Moreover, substitution of aspartate for either of the glycines immediately upstream from the terminal cysteines greatly diminished

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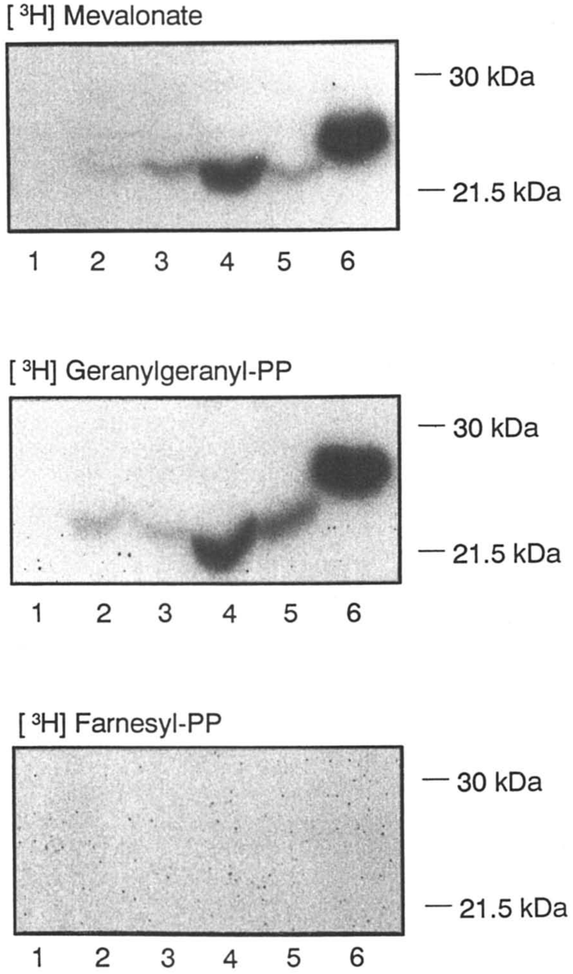
[^{3}H] Mevalonate
— 30 kDa
— 21.5 kDa
1 2 3 4 5 6
[^{3}H] Geranylgeranyl-PP
— 30 kDa
— 21.5 kDa
1 2 3 4 5 6
[^{3}H] Farnesyl-PP
— 30 kDa
— 21.5 kDa
1 2 3 4 5 6

Figure 3. Isoprenylation of *rab* proteins *in vitro*. RNA transcripts of *rab*1B, *rab*2, *rab*4, *rab*5 and *rab*6 were translated in a rabbit reticulocyte lysate system containing either [^{3}H]mevalonate, [^{3}H]farnesyl pyrophosphate, or [^{3}H]geranylgeranyl pyrophosphate (see refs. 25 & 26 for detailed methods). Parallel translations without added RNA served as controls. Aliquots from each reaction (12.5 μl) were subjected to SDS-PAGE and fluorography. Samples were as follows: -RNA (lane 1); *rab*4 (lane 2); *rab*6 (lane 3), *rab*1B (lane 4), *rab*2 (lane 5); *rab*5 (lane 6). The mobilities of the 21.5 kDa and 30 kDa standard proteins are indicated at the right of each panel.

isoprenylation, suggesting that these glycines form an essential part of the structure recognized by the isoprenyltransferase. In contrast, substitution of arginine for the serine in the fifth position from the carboxyl terminus had no effect on isoprenylation. These results indicate that isoprenylation of the proteins of the *rab*1/*rab*2/ YPT1 type requires an intact G-G-C-C sequence at the carboxyl terminus. However, a number of questions remain to be answered. For instance, are both cysteines in the G-G-C-C sequence actually modified, or does one simply serve as part of the recognition motif for the other? Are conservative substitutions (*e.g.*, alanine) permitted in the positions occupied by the glycines? Are structural domains beyond the carboxyl terminus involved in recognition by the isoprenyltransferase? Do the *rab* proteins undergo additional modifications (*e.g.*, carboxylmethylation) similar to those described for the *ras* proteins? Although these questions remain unanswered in the case of the *rab*1 and *rab*2 proteins, a recent study of a proteolytically derived fragment corresponding to the carboxyl terminus of purified brain *rab*3/smg25A protein indicates that both cysteines in the *x*-C-*x*-C motif are modified by geranylgeranyl and that the terminal cysteine is methylesterified (*59*). It is not yet known whether the amino acids occupying the *x* positions in the *x*-C-*x*-C motif play a role in recognition by the geranylgeranyl transferase, but the residues occupying these positions in *rab*3A (D-C-A-C), *rab*4 (E-C-G-C) and *rab*6 (G-C-S-C) do not suggest an obvious pattern.

TABLE II. Effects of carboxyl-terminal alterations on isoprenylation of *rab*1B protein

Amino Acid Sequence[a]	*Relative $[^3H]$Mevalonate Incorporation*[b]
(wt) A-S-G-G-C-C *	++++
A-S-G-G-C $\underline{\ast}$	--
A-S-G-G $\underline{\ast}$	--
A-S-G-G-C-$\underline{S}$ *	+
A-S-G-G-$\underline{S}$-C *	+
A-S-G-$\underline{D}$-C-C *	--
A-S-$\underline{D}$-G-C-C *	+
A-$\underline{R}$-G-G-C-C *	++++

*Represents carboxyl terminus.

[a] Alterations introduced by site-directed mutagenesis are underlined.

[b] See ref. *25* for actual fluorograms and incorporation values.

(Adapted from ref. *25* with permission, copyright 1991.)

Genetic Evidence for Multiple Protein:Isoprenyltransferases

Direct biochemical evidence cited above indicates that mammalian cells contain at least two distict protein:isoprenyltransferases, one that catalyzes the transfer of farnesyl to proteins ending with a carboxyl-terminal C-*a*-*a*-S/M

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motif, and another that catalyzes the transfer of geranylgeranyl to proteins ending with a C-*a*-*a*-L motif. The recent finding that *rab* proteins ending with *x*-C-*x*-C and G-G-C-C sequences can undergo geranylgeranyl modification implies that cells may contain additional unique protein:isoprenyltransferases with specificities directed toward each of these distict carboxyl-terminal motifs.

The existence of multiple protein:isoprenyltransferases in eucaryotic cells has been confirmed by an independent line of evidence derived from genetic studies in yeast. Farnesylation of the **a**-mating factor and *RAS* proteins in *Saccharomyces cerevisiae* has been shown to depend on the product of the *DPR1/RAM1* gene (*34,60*), whereas the activity of a geranylgeranyltransferase that modifies proteins with a C-*a*-*a*-L motif depends on the product of the *CDC43/CAL1* gene (*61,62*). In complementation assays, the *DPR1/RAM1* gene product cannot compensate for mutations in the *CDC43/CAL1* locus. Therefore, the products of the *DPR1/RAM1* and *CDC43/CAL1* loci may correspond repectively to the unique β subunits of the mammalian farnesyl and geranylgeranyl transferases. Since both isoprenyltransferase activities are affected in yeast strains with mutations in the *RAM2* locus (*60,61*), the *RAM2* gene product could represent the yeast homolog of the common α subunit of the mammalian farnesyl and geranylgeranyl transferases (*51*). Most recently, Rossi *et al.* (*63*) have reported that membrane attachment of the YPT1 and SEC4 proteins depends on the product of the *BET2/ORF2* gene. Since the predicted sequence of the *BET2* protein shares some homology with the products of the *DPR1/RAM1* and *CDC43/CAL1* genes (*62,63*), it seems likely that the protein could be a unique subunit of a geranylgeranyltransferase with specificity directed toward proteins with the G-G-C-C carboxyl terminal structure. Ongoing studies of the yeast protein:isoprenyltransferase genes undoubtedly will provide valuable clues concerning the diversity of their mammalian counterparts.

Future Directions

The isolation and characterization of the enzymatic systems that catalyze protein isoprenylation (*47-52*) will accelerate progress toward understanding how the low molecular mass GTP-binding proteins function in a variety of physiological processes, including signal transduction, growth regulation, secretion, and endocytosis (*32,33,53*). Studies of oncogenic variants of p21^{ras} have already established that farnesylation is an essential step for the expression of *ras* transforming activity (*23,31,36*). With increasing knowledge of the structural features recognized by the p21^{ras}:farnesyltransferase, the prospect of devising new approaches to cancer therapy based on peptide inhibitors of this enzyme has attracted considerable attention (reviewed in ref. *65*). However, since the nuclear lamins (*11,64*) and possibly other unidentified farnesylated proteins (see Figure 1) may be modified by the same enzyme, important questions about the specificity of any such therapeutic approach will have to be answered.

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In the case of the newly discovered geranylgeranyl modification of the *rab* proteins, it is reasonable to speculate that the isoprenoid modification may underlie the ability of these proteins to associate with discrete cellular membrane systems, such as the endoplasmic reticulum, Golgi complex and endosomes. However, because of the specificity and apparent reversibility of these membrane associations (*53*), it seems likely that other factors, such as protein-protein interactions, also play an important role in targeting *rab* proteins to particular membranes. Although recognition of *rab*1B protein by geranylgeranyltransferase appears to involve at least two amino acids upstream from the carboxyl-terminal cysteines (see Table II), our preliminary studies with pentapeptides based on the carboxyl-terminal sequences of the *rab*1B and *rab*6 proteins (Kinsella & Maltese, unpublished) have failed to show competition for prenylation of the corresponding recombinant proteins, similar to that demonstrated in the case of $p21^{ras}$:farnesyltransferase (*47,49*). Therefore, the recognition signals for isoprenylation of the *rab* proteins may turn out to be relatively complex, possibly involving amino acids at some distance from the carboxyl-terminus.

The metabolic regulation of the various protein:isoprenyltransferases in mammalian cells has not yet been explored. Preliminary evidence suggests that the activity of the geranylgeranyltransferase involved in modifying proteins with a C-*a*-*a*-L motif does not undergo regulation by sterols, unlike other enzymes in the isoprenoid biosynthetic pathway (3-hydroxy-3-methylglutaryl-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA synthase and farnesyl pyrophosphate synthase) (*66*). However, changes in the activity of the geranylgeranyltransferase apparently occur during brain development (*66*), suggesting that the regulation of protein:isoprenyltransferases in relation to cell growth and differentiation may be a worthwhile subject for future investigation. The pace of research in this exciting area will certainly increase as the genes coding for the mammalian protein:isoprenyltransferases are cloned and antisera to specific enzyme subunits become available.

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Chapter 7

Potent, Rationally Designed Inhibitors of Squalene Synthase

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The first class of potent inhibitors of squalene synthase is described. The lead inhibitor, ether **10**, was discovered as a result of rational design on the basis of a proposed mechanism for the enzymatic reaction. The ether oxygen was positioned to hydrogen bond with a putative active site acid catalyst and was found to make a substantial contribution to the inhibitor-enzyme binding energy. The structural requirements for potent inhibition are discussed.

With the discovery of the mevinic acid family of HMG-CoA reductase inhibitors, the inhibition of *de novo* cholesterol biosynthesis has been validated as a method for the treatment of hypercholesterolemia in humans *(1-3)*. Three members of this family, lovastatin, pravastatin and simvastatin, are currently in clinical use. In addition, a large number of synthetic and semisynthetic analogues have been reported and are in various stages of development *(4-8)*. Although HMG-CoA reductase inhibitors are safe and highly efficacious drugs *(2, 3)*, the study of alternative targets in the cholesterol biosynthetic pathway should provide additional insight into the modulation of lipid homeostasis.

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We proposed *(9-11)* that the inhibition of squalene synthase may have advantages over intervention earlier in the cholesterol biosynthetic pathway (**Figure 1**). Squalene synthase *(12-14)* catalyzes the reductive dimerization of farnesyl diphosphate (**1**, FPP) to squalene (**2**). This enzyme occupies a strategic site at the final branch point in the pathway. Selective inhibition of squalene synthase should not directly suppress the production of the non-sterol isoprene metabolites including ubiquinone, dolichol, isopentenyl *t*-RNA and isoprenylated proteins. This chapter describes our progress towards the design, synthesis and evaluation of squalene synthase inhibitors *(9-11, 15)*. Our ultimate goal is the discovery of a superior agent for treatment of hypercholesterolemia.

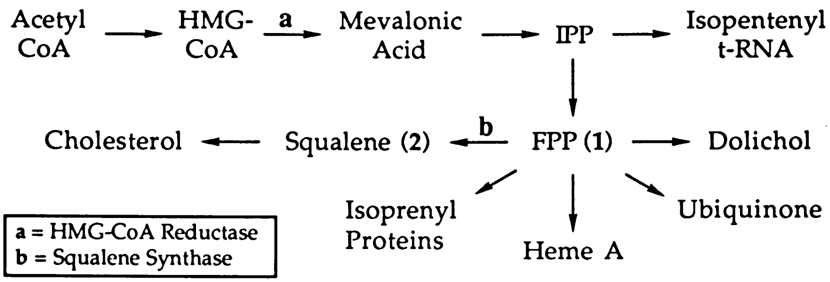

Figure 1. Isoprene Biosynthesis Pathway: an abbreviated representation of the biosynthetic routes to cholesterol and the non-sterol isoprenes.

Enzymology. Squalene synthase is a microsomal protein and is typically studied as a crude microsomal preparation from liver or yeast *(12-14)*. We have developed an assay for squalene synthase activity which utilizes the 100,000 x g microsomal pellet derived from rat liver homogenate as a source of enzyme and gas chromatography to quantitate squalene synthesis from unlabeled FPP substrate *(9, 10)*. The production of squalene is more commonly monitored by the incorporation of radiolabel from ^{3}H- or ^{14}C-FPP *(12, 14)*. Divalent metal ion (Mg^{+2} or Mn^{+2}) and reduced pyridine nucleotide (NADH or NADPH) are required for the enzymatic reaction.

The purification of squalene synthase in soluble form has been a challenge. Recently, Rilling and coworkers *(16)* succeeded in purifying solubilized squalene synthase from yeast to homogeneity. Nonionic detergents, methanol and sucrose were found to enhance the stability of the soluble enzyme. Two research groups reported the cloning and expression of the squalene synthase gene from the yeast *Saccharomyces cerevisiae (17, 18)*. The gene codes for a protein of 444 amino acids (M_r = 51.7 kD). The C-terminal hydrophobic domain was identified as a likely membrane spanning region which serves to anchor the protein in the endoplasmic reticulum. Squalene synthase activity is coordinately regulated *(19)* by intracellular sterol concentrations *(20-22)*, in a manner similar to HMG-CoA reductase.

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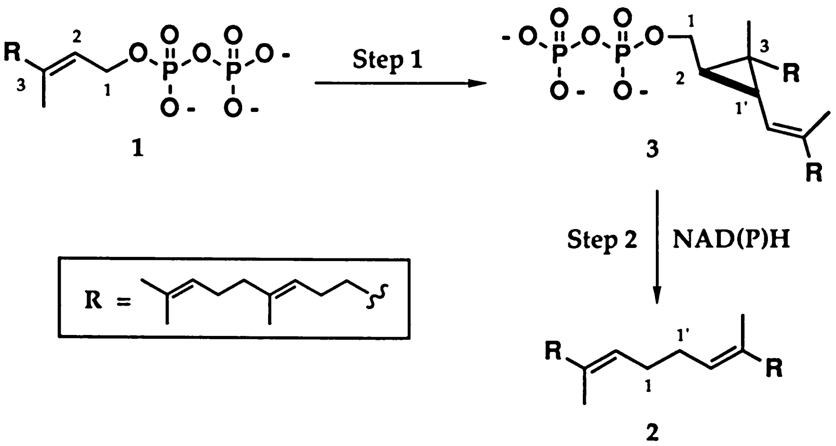
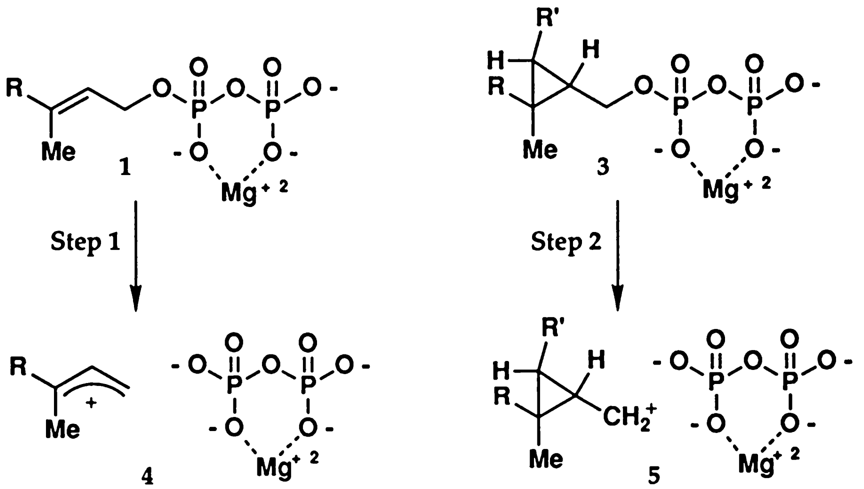
Reaction Mechanism. The squalene synthase reaction is a two step transformation which proceeds through the intermediate cyclopropane, presqualene diphosphate (**3**, **Scheme 1**) *(23)*. The soluble, purified enzyme *(16)* and the recombinant enzyme *(17, 18)* from yeast produce both presqualene diphosphate and squalene, indicating that the two transformations are catalyzed by a single protein. In the first step *(12, 13)*, one equivalent of FPP (the prenyl donor) undergoes a loss of the *pro-S*-hydrogen and inorganic diphosphate (PP_i) from C-1, concomitant with the insertion of C-1 into the C-2/C-3 alkene of the second equivalent of FPP (the prenyl acceptor) to form **3**. In the subsequent step *(12, 13, 24)*, presqualene diphosphate undergoes a reductive rearrangement in which a second molecule of PP_i is released and NAD(P)H is consumed to form the symmetrical dimer, squalene. The donor and acceptor binding sites exhibit independent selectivity for the acceptance of modified FPP analogues as substrates, indicating that the two sites are distinct *(25, 26)*.

Scheme 1



It has been proposed that the initial process involved in both steps of the squalene synthase reaction is heterolysis of the C-OPP_i bond to form an ion pair (**Scheme 2**) *(12, 24, 27)*. In the case of step 1, allyl cation **4** is produced, whereas in the case of step 2, the cyclopropyl carbinyl cation **5** is formed. This proposal is supported by chemical model studies for step 2 as well as elegant inhibition studies involving mimics of ion pair **5** *(12, 24, 27)*. Several lines of evidence indicate that the closely related prenyl transferase reaction catalyzed by FPP synthase proceeds *via* allyl ion pairs analogous to **4** *(27-31)*. In addition, heterolysis of diphosphate esters to form stabilized carbonium ions is a documented aspect of the intrinsic chemistry of this functional group *(32)*. The studies described in this chapter center on the initial process of ion pair formation. Space does not allow for discussion of the fascinating mechanistic transformations subsequent to the initial step and the reader is referred to several key reviews *(12-14, 24, 27)*.

Scheme 2



1 3 4 5

Diphosphate Surrogates

In the design of inhibitors of squalene synthase based on the structure of substrate **1**, as well as related transition states and intermediates, stable surrogates for the diphosphate moiety have to be devised. Although the diphosphate is crucial for binding and catalysis, it is unsuitable as an inhibitor component due to its chemical (high and low pH) *(32)* and biological (phosphatases) lability. We have evaluated a number of potential diphosphate surrogates *(9-11)*, and concentrate here on the (phosphinylmethyl)phosphonate (PMP) moiety *(9, 15)*. Parent PMP **6a** is simply the result of replacing both the reactive allylic and anhydride oxygens of FPP with CH_2 *(9)*. This modification affords a stable molecule which is unable to undergo the initial step of the squalene synthase reaction. *A priori*, it was difficult to predict whether either of these two oxygens was crucial for interaction with the enzyme. Upon evaluation in the rat liver microsomal squalene synthase assay, **6a** proved to be an effective inhibitor of the enzyme, $I_{50} = 31.5\ \mu M$ (**Table 1**). Lineweaver-Burk analysis indicates that **6a** is competitive with respect to FPP with $K_i = 10\ \mu M$ (**Figure 2A**). Comparison with the Michaelis constant for FPP under our assay conditions (apparent $K_m = 12.7\ \mu M$) reveals that **6a** and FPP bind to the enzyme with similar affinity. We infer from this that neither the anhydride nor allylic oxygens of FPP are critical for substrate binding.

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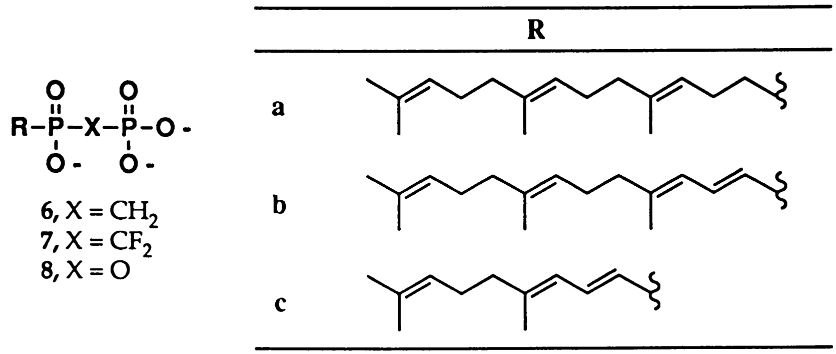

6, X = CH_2
7, X = CF_2
8, X = O

Table 1. Inhibition of Squalene Synthase by Compounds 6, 7 and 8*

Cpd	I_{50} (μM)	Cpd	I_{50} (μM)
6a	31.5	7a	29.9
6b	12.2	7b	15.5
6c	-40 % @ 600	8a	42

*For assay conditions, see references 9 and 10.

The results of our initial study on PMP inhibitors related to **6a** are presented in **Table 1**. 1,3-Dienyl PMP **6b** exhibited improved activity relative to **6a**, suggesting that a vinyl group may be a superior substitute for the allylic C-O bond. The difluoro-PMP surrogate of **7a** and **7b** was intended to more closely mimic the diphosphate of FPP with respect to pK_a values *(33)*, thereby favoring the fully ionized form at physiological pH. The equivalent inhibitory potencies of the corresponding PCH_2P (**6a, 6b**) and PCF_2P (**7a, 7b**) analogues indicate that both **6** and **7** can achieve the protonation state necessary for binding to the enzyme. The geranyl derivative **6c** is at least 20-fold less potent than its farnesyl counterpart **6b**. This is consistent with reports that geranyl diphosphate is not a substrate *(34)* and is a poor inhibitor *(35)* for squalene synthase. Inhibitor **8a** *(36)*, an

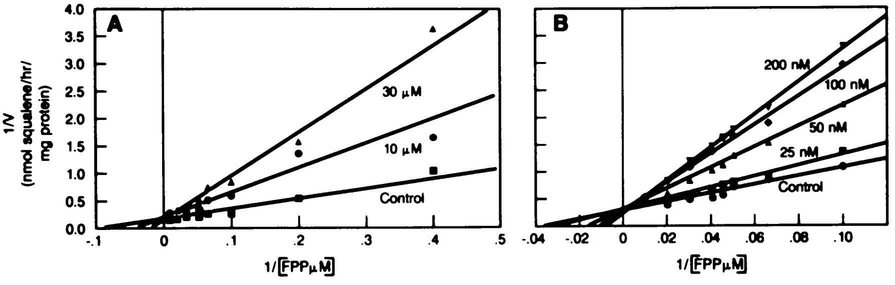

Figure 2. Lineweaver - Burk analysis of the inhibition of rat liver microsomal squalene synthase by **6a** (**Figure 2A**) and **10** (**Figure 2B**).

analogue which retains the anhydride linkage, possesses activity comparable to **6a**, providing further evidence that the anhydride oxygen is not essential. In related work *(37)*, Poulter, McClard and coworkers demonstrate that the PMP analogues of isopentenyl- and dimethylallyl diphosphate are inhibitors of the prenyl transferase, FPP synthase.

Rational Design of Potent PMP Inhibitors

Rationale. PMP inhibitors **6a,b** and **7a,b** bind to squalene synthase with an affinity which is comparable to that of the substrate FPP and thus represent a starting point for further studies. As a basis for rational design, we focused on a hypothetical model for FPP binding to the donor site of the enzyme and subsequent ion-pair formation (**Figure 3**). We envision that

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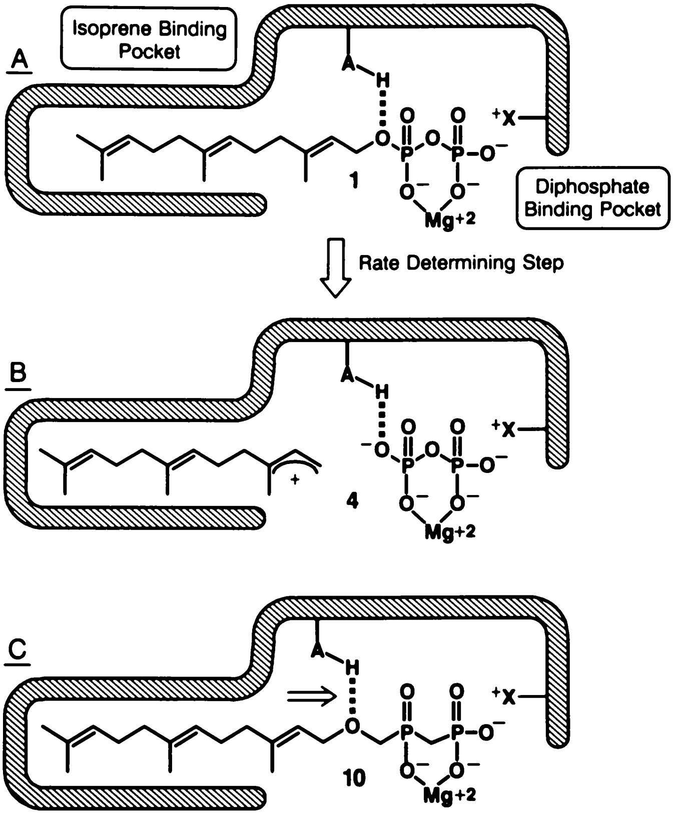

Figure 3. Hypothetical model of the donor site of squalene synthase, bound to: (**A**) substrate FPP, (**B**) the allyl cation - diphosphate ion pair **4** and (**C**) ether inhibitor **10**. The active site function A-H represents the putative acid catalyst and X^+ represents cationic side chain(s) which ion pair with the phosphate groups.

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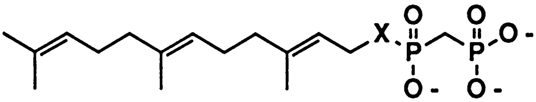
FPP is bound to the donor site of squalene synthase *via* hydrophobic interactions with the isoprene subunit and ionic interactions with the highly charged diphosphate moiety (**Figure 3A**). We propose two mechanisms by which the enzyme might catalyze the generation of ion-pair **4** from enzyme bound **1**. First, we expect that the enzyme interacts more strongly with the two fragments formed in the conversion of **1** to **4** as the distance between them approaches the transition-state separation. The enzyme might accomplish this by positioning the isoprene and diphosphate binding pockets such that optimal binding energies are achieved at the transition-state distance. In this manner, the active site serves to *stretch* the C-OPP_i bond. Second, we propose that squalene synthase utilizes an active site acid catalyst to promote ion-pair formation (A-H in Figure 3). This acid is expected to interact weakly with substrate **1** due to the poor basicity of the ester oxygen, but much more strongly as the basic phosphate anion of **4** is formed. Identical arguments can be applied to the formation of ion-pair **5** from **3**.

Following the above analysis, we attempted to create more potent PMP inhibitors by: (1) increasing the separation between the isoprenyl subunit and the PMP surrogate in order to approximate the ion pair separation and/or (2) incorporating functions in the linking chain to interact with the putative active site acid. The former may be difficult to accomplish, since we are restricted to normal covalent bond lengths, a limitation not shared by transition states.

Results. Linker homolog **6d** *(9)*, an attempt to approximate the transition state separation between the fragments, is actually a somewhat weaker inhibitor of the enzyme than **6a** (**Table 2**). Incorporation of a phosphonate ester oxygen atom in the linking chain to interact with the putative active site acid (example **9**) results in a 4-fold increase in activity relative to **6d**. This modest improvement is not surprising, since an ester oxygen, like that of FPP itself, is expected to form a relatively weak hydrogen bond with

Table 2. Linker Modifications of the PMP Inhibitors*



Cpd	Linker X	I_{50} (μM)	Cpd	Linker X	I_{50} (μM)
6a	$-CH_2-$	31.5	**12**	$-CH_2OCH_2-$	0.15
6d	$-CH_2CH_2-$	67.0	**13**	$-CH_2CH_2OCH_2-$	79.3
9	$-CH_2O-$	16.0	**14**	$-NH_2^+CH_2-$	16.0
10	$-OCH_2-$	0.05	**15**	$-SCH_2-$	122
11	$-OCH_2CH_2-$	2.3			

*For assay conditions, see references 9 and 10.

the acid moiety. When a more basic ether oxygen was inserted in the linking chain (example **10**), a profound increase in inhibitory potency resulted *(15)*. Ether **10** (I_{50} = 0.05 μM) is 1340-fold more potent than its carbon isostere **6d** as an inhibitor of squalene synthase! Inhibitor **10** was demonstrated to be competitive with respect to FPP, K_i = 0.037 μM **(Figure 2B)**.

Further modifications of the linking region *(15)* were aimed at revealing structural features that are important for inhibition **(Table 2)**. The position of the ether oxygen relative to the phosphinic acid is critical. Insertion of an additional CH_2 between the ether and phosphinic acid results in a 46-fold loss in activity (example **11**), whereas homologation on the other side of the ether (example **12**) leads to just a 3-fold loss. Further homologation of **12** to **13**, however, is highly deleterious. Consistent with the requirement for a hydrogen bond acceptor is the unimpressive inhibitory potencies of ammonium ion **14**, which is an H-bond donor, and thioether **15**, which is a poor H-bond acceptor.

Enzyme-Inhibitor Binding Energy

It would be useful for future work in the area of molecular recognition to arrive at a quantitative accounting of the contribution made by the ether oxygen to enzyme-inhibitor interactions. In order to accomplish this, we compare ether **10** to its carbon isostere **6d** **(Figure 4)**. The difference between the enzyme binding energies of **10** and **6d** in aqueous solution ($\Delta\Delta G_{water}$) is calculated to be 4.3 kcal/mol utilizing the expression: $\Delta\Delta G_{water} = -RT\ln[I_{50}(\mathbf{10})/I_{50}(\mathbf{6d})]$. The differential binding energy in the absence of solvent ($\Delta\Delta G_{gas}$) *(38)* is a more meaningful measure of the difference between the two inhibitors, since, by factoring out solvation, $\Delta\Delta G_{gas}$ is solely a function of the interactions within the E-I complex.

Using the thermodynamic cycle in **Figure 4** as suggested by the work of Bartlett *(38-41)*, the expression in **eq 1** for $\Delta\Delta G_{water}$ is derived. Solving for $\Delta\Delta G_{gas}$ affords **eq 2**, since $\Delta\Delta G_{solv\text{-}E}$ is zero. To further simplify this expression *(38)*, we make the assumption that the term $\Delta\Delta G_{solv\text{-}E\text{-}I}$ is also zero, since it is likely that water is excluded from the binding site of both E-I complexes. If anything, this approximation should lead to an underestimation of $\Delta\Delta G_{gas}$, since the solvation energy of **E-6d** has the potential to be larger than that of **E-10** in the event that water is accessible to the active site functionality (*eg.* the putative acid catalyst) which is satisfied by the ether in the case of **E-10**. Thus, $\Delta\Delta G_{gas}$ is approximated as the sum of $\Delta\Delta G_{water}$ (4.3 kcal/mol) and $\Delta\Delta G_{solv\text{-}I}$, where $\Delta\Delta G_{solv\text{-}I}$ is the difference between the solvation energies of **10** and **6d**.

According to the empirical correlations of Hine *(42)*, an ether oxygen contributes 4.2 kcal/mol more than a CH_2 to the solvation energy. Thus, $\Delta\Delta G_{gas}$ for **10** relative to **6d** is estimated to be 8.5 kcal/mol. In water, approximately half of this energy is expended for the desolvation of the ether function. Assuming that **10** and **6d** bind to squalene synthase in the same manner, we speculate that this large differential binding energy is due to hydrogen bonding of the ether oxygen to an active-site acid catalyst

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as illustrated in **Figure 3-C**. The ether oxygen is of intermediate basicity relative to the weakly basic phosphate ester oxygen in **Figure 3-A** and the strongly basic phosphate anion in **Figure 3-B**, and can be considered to be a mimic of a species along the reaction coordinate from **A** to **B**. Large $\Delta\Delta G_{gas}$ have been observed previously for isosteric functional group replacements in inhibitors of thermolysin *(38, 39)* and nucleoside deaminases *(40, 41)*.

$$\begin{tikzcd}
E_{water} \arrow[d, "-\Delta G_{solv\text{-}E}"'] & + & I_{water} \arrow[d, "-\Delta G_{solv\text{-}I}"] \arrow[r, "\Delta G_{water}"] & E\text{-}I_{water} \\
E_{gas} & + & I_{gas} \arrow[r, "\Delta G_{gas}"] & E\text{-}I_{gas} \arrow[u, "\Delta G_{solv\text{-}E\text{-}I}"']
\end{tikzcd}$$

$$\Delta\Delta G_{water} = -\Delta\Delta G_{solv\text{-}E} - \Delta\Delta G_{solv\text{-}I} + \Delta\Delta G_{gas} + \Delta\Delta G_{solv\text{-}E\text{-}I} \quad \text{(eq 1)}$$

$$\Delta\Delta G_{gas} = \Delta\Delta G_{water} + \Delta\Delta G_{solv\text{-}I} - \Delta\Delta G_{solv\text{-}E\text{-}I} \quad \text{(eq 2)}$$

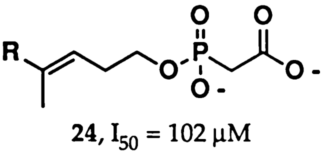
$$= 4.3 + 4.2 + 0 = 8.5 \text{ kcal/mol}$$

Figure 4. Thermodynamic cycle is utilized to estimate the differential binding energy of **10** relative to **6d** in the absence of solvent ($\Delta\Delta G_{gas}$).

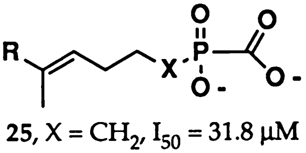
An alternative explanation for the large differential binding energy is an indirect effect of the ether on the interaction of the PMP surrogate with the enzyme. If anything, the electron withdrawing capabilities of the ether would be expected to decrease the binding energy of the adjacent phosphinic acid group *(38, 43)*. Again, this would lead to an underestimation of the contribution made by the ether.

Further Structure-Activity Studies

Modifications of the PMP Diphosphate Surrogate. Studies were undertaken to determine whether the complete triacid PMP surrogate was essential for potent inhibition. The strong interaction offered by the ether does not compensate for the loss of either phosphorus moiety, as evidenced by the poor activity exhibited by **16** and **17** (**Table 3**). Further attempts to reduce the triacid PMP surrogate of **10** to a diacid resulted in considerable loss of potency as evidenced by examples **18** - **23**. In the case of phosphinyl acetate



24, I_{50} = 102 µM



25, X = CH_2, I_{50} = 31.8 µM
26, X = O, I_{50} = 8.7 µM

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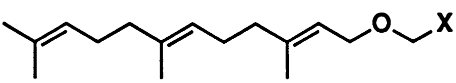
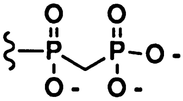
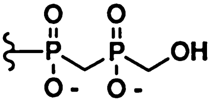
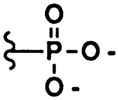
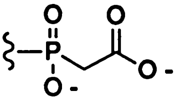
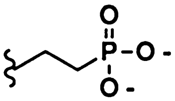
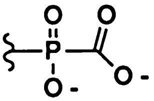
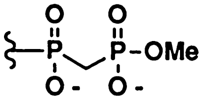
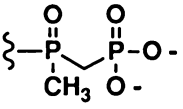
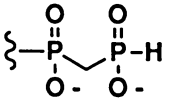
Table 3. Modifications of the PMP Diphosphate Surrogate*



Cpd	X	I_{50} (μM)	Cpd	X	I_{50} (μM)
10		0.05	20		174
16		92	21		12.9
17		Inactive @ 300	22		87.4
18		Inactive @ 300	23		94
19		75			

*For assay conditions, see references 9 and 10.

21, the ether analogue is 8-fold more potent than the corresponding ester linked compound 24; however, this is a minor improvement compared to the same modification in the PMP series (9 *vs* 10). Phosphinyl formate 22 is actually less potent than isosteres 25 and 26 *(11)*, suggesting that the phosphinyl formates bind either to a different site on the enzyme (*eg*, the acceptor site), or to the same site but in a different manner.

Modification of the Isoprene Subunit. The results of our studies on the modification of the farnesyl subunit of 10 are presented in **Table 4.** Beginning with the terminal isoprene unit, reduction of the olefin (example 27) and further removal of a CH_3 group (example 28) both led to > 10-fold loss in potency relative to 10. These same modifications to FPP itself have been reported to afford good substrates for squalene synthase *(26, 34)*. Deletion of the entire terminal isoprene unit (geranyl analogue 29) effects an almost complete loss of inhibitory activity. This is consistent with our prior observation with geranyl PMP 6c *(vide supra)*, and confirms the requirement for a complete farnesyl chain-length for strong enzyme-inhibitor binding.

The enzyme is more tolerant of structural variations in the isoprene unit proximal to the ether. Removal of the vinylic methyl group (example

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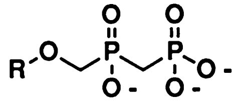
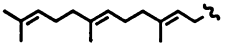
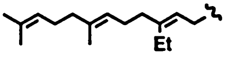
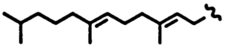
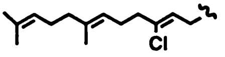
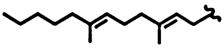
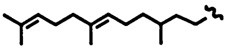
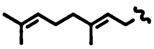
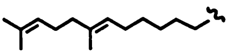
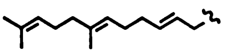
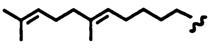
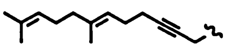
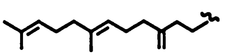
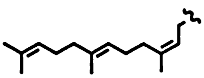
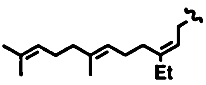
30), homologation to an ethyl group (example 33) and replacement with chloride (example 34) all result in minimal loss of potency. When the first two modifications are made to FPP itself, the resulting allylic diphosphates are reported to be extremely poor substrates for the enzyme *(25, 26)*. Surprisingly, replacing the proximal *(E)*-olefin with an acetylene (example 31) or a *(Z)*-olefin (example 32) leads to complete retention of inhibitory activity. Molecular modeling studies suggest that both **31** and **32** can attain conformations that overlap with **10** at both the terminal isoprene unit and the ether-PMP portions of the molecule, while bridging the two termini *via* different routes. These results indicate that there is an element of structural flexibility in the interior region of the inhibitor.

Table 4. Modifications of the Isoprenyl Subunit*



Cpd	R	I_{50} (μM)	Cpd	R	I_{50} (μM)
10		0.05	33		0.078
27		0.53	34		0.107
28		0.59	35		0.155
29		> 300	36		0.315
30		0.17	37		0.218
31		0.066	38		0.412
32		0.047	39		0.178

*For assay conditions, see references 9 and 10.

Summary of SAR Studies. The results of our SAR studies on PMP inhibitors of squalene synthase are summarized in **Figure 5**. The requirements for potent enzyme inhibition are: (1) an intact, triacid PMP surrogate, (2) an ether oxygen adjacent to the PMP surrogate, and (3) a farnesyl-length isoprene subunit. A certain degree of structural flexibility is allowed in the isoprene subunit that is proximal to the ether.

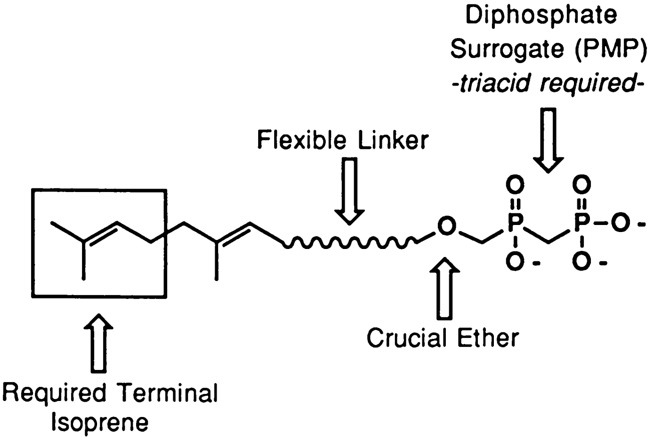


Figure 5. Anatomy of a Squalene Synthase Inhibitor

Further Biological Evaluation of 10

The inhibition of squalene synthase by inhibitor **10** in human liver and yeast microsomes was compared to that in rat liver microsomes under our standard assay conditions *(9, 10)* (**Table 5**). Although the results from both mammalian sources were nearly identical, **10** is much less effective against the yeast enzyme. The apparent K_m values for FPP in the rat (12.7 μM) and yeast (25 μM) microsomal systems are similar. Upon expressing the relative potency for the two enzyme preparations as $(K_m/K_i)_{rat}/(K_m/K_i)_{yeast}$, **10** is demonstrated to be 16-fold more active against the rat *vs* the yeast enzyme. This may be an indication that the active sites of the yeast and mammalian enzymes are significantly different. In addition, **10** inhibits cholesterol biosynthesis from ^{14}C-acetate in whole, freshly-isolated hepatocytes (I_{50} = 2.7 μM). The parent PMP analogue **6a** is inactive in the whole cell assay at up to 100 μM *(11)*.

Table 5. Effect of **10** on Squalene Biosynthesis in Microsomes and on Cholesterol Biosynthesis in Rat Hepatocytes

Assay	I_{50} (μM)	K_i
Rat Liver Microsomes[a]	0.05	0.037
Human Liver Microsomes[a,b]	0.038	----
Yeast Microsomes[a,c]	2.6	1.18
Whole Rat Hepatocytes[d]	2.7	----

(a) For standard assay conditions, see refs 9 and 10. (b) From frozen human liver. (c) Recombinant, overexpressed enzyme *(17)*. (d) For assay, see ref. 11.

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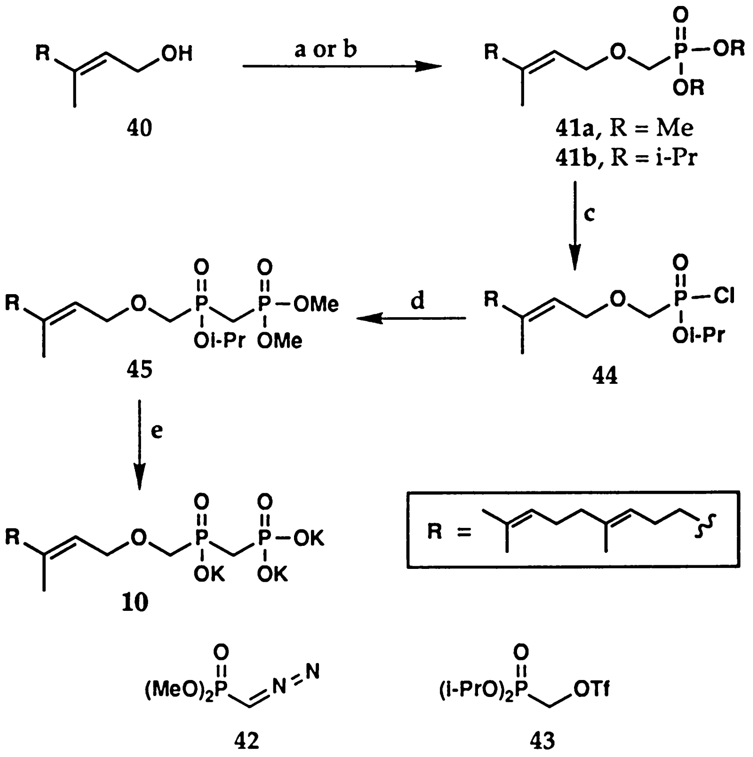
Synthesis of Inhibitor 10

The synthetic route to ether-PMP **10** (Scheme 3) is general and was utilized to prepare most of the inhibitors described herein. Two methods for the preparation of alkoxy phosphonate **41** from farnesol **40** were evaluated. Rhodium-catalyzed carbene insertion *(44)* of diazomethylphosphonate **42** *(45)* provided dimethyl ester **41a** in modest yield. The reaction of the lithium alkoxide derived from farnesol with triflate **43** *(46)* afforded the corresponding diisopropyl ester **41b** in 92 % yield. Triflate **43** is prepared from diisopropyl phosphite in two steps (paraformaldehyde, Et_3N, 100 °C *(47)*; trifluoromethanesulfonic anhydride, $i\text{-}Pr_2NEt$, ether, -78 to 0 °C, 52 % overall). Either **41a** or **41b** can be used to complete the synthesis following our previously reported methodology for the preparation of PMP inhibitors *(48)*. In the case of **41b**, conversion to acid chloride **44** was

Scheme 3



(a) 0.2 equiv $Rh_2(OAc)_2$, 2 equiv **42**, PhH, RT, 36 %. (b) BuLi, THF, -78 °C; **43**, 0 °C, 92 %. (c) KOH, H_2O, i-PrOH, 100 °C; $TMSNEt_2$, CH_2Cl_2, oxalyl chloride, cat DMF, CH_2Cl_2. (d) $LiCH_2PO(OMe)_2$, THF, -78 °C, 79% overall from **41b**. (e) TMSBr, CH_2Cl_2, 2,4,6-collidine; KOH, H_2O, 93 %.

followed by coupling with the lithium anion of dimethyl methylphosphonate to provide PMP triester **45** (79 % yield from **41b**). Bromotrimethylsilane *(49)* promoted deesterification gave inhibitor **10** in 93 % yield.

Conclusion

In summary, our studies have led to the first family of potent inhibitors of squalene synthase. Inhibitor **10** was discovered as the result of rational design on the basis of a proposed mechanism for the enzymatic catalysis of squalene biosynthesis. This study revealed a surprisingly large contribution of an ether oxygen to the inhibitor-enzyme binding energy. We speculate that this oxygen is engaged in a hydrogen bonding interaction with a key active site acid catalyst. Further structure-activity studies reveal that a triacid diphosphate surrogate and a farnesyl-length isoprenyl subunit are required for optimal inhibitory activity, and that a degree of structural flexibility is tolerated in the isoprenyl portion of the inhibitor.

Acknowledgements. The authors would like to thank Drs. Philip M. Sher, William H. Koster and Richard E. Gregg for their helpful comments on this manuscript, Dr. Val S. Goodfellow for assistance with the molecular modeling studies, and Dr. Jeffrey M. Hoeg of the National Institutes of Health for a sample of human liver tissue.

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Chapter 8

Oxysterol Regulation of Cholesterol Biosynthesis

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This review is focused upon the oxysterol model for the regulation of cholesterol biosynthesis. This model postulates that oxygenated derivatives of cholesterol or lanosterol are produced in cells as signal molecules which bind to an oxysterol binding protein (receptor) and repress cholesterogenic gene expression. Progress made in identifying the major elements of the oxysterol model, i.e. a candidate oxysterol receptor protein and candidate endogenous oxysterol regulators is summarized, and critical, unresolved issues in the oxysterol hypothesis are presented.

The oxysterol model (*1*) for the regulation of cholesterol biosynthesis proposes that oxygenated derivatives of cholesterol or lanosterol are produced in cells as signal molecules which feedback regulate enzymes of the cholesterol biosynthetic pathway. It is further proposed that these oxysterols bind to an oxysterol binding protein (receptor) which then mediates the repression of cholesterogenic gene transcription. The model is founded on the discovery that certain oxysterols act specifically to repress cholesterogenic gene transcription when added to mammalian cell cultures. In addition, evidence has been found for the two major elements of the model—an intracellular receptor protein and endogenous regulatory oxysterols. Conclusive proof for the regulatory roles of these elements is not yet available, nor is it known how oxysterol concentrations are controlled in cells or how the oxysterol receptor mediates the regulatory response.

In addition to feedback repression, cholesterogenic gene expression is greatly affected in some tissues by developmental and hormonal changes (2) which may occur by independent mechanisms (*3, 4*). The activity of one enzyme early in the pathway, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), may be regulated at a number of levels including phosphorylation (*5*), degradation (*6, 7*), sulfhydryl oxidation (*8*), message translation (*9*) and message stability (*3*). The sites and circumstances where oxysterol control is of physiological significance is another question that must be resolved by studies of this regulatory pathway.

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Here I briefly review the background of the oxysterol model and the current efforts to resolve these central issues.

Background of the Oxysterol Hypothesis

It was discovered in the early 1950's that dietary cholesterol represses hepatic cholesterol synthesis in experimental animals. However, since other, metabolically unrelated steroids also repressed hepatic sterol synthesis when presented in the diet, it could not be concluded that cholesterol *per se* was the ultimate feedback regulator (*10*). Investigating this issue in cell cultures, Kandutsch and Chen (*11*) found that addition of freshly recrystallized cholesterol to the culture medium did not repress de novo cholesterol synthesis. The failure of pure cholesterol to regulate synthesis in cell cultures was not due to a lack of uptake since the concentrations of free sterol in three different cell types increased two- to seven-fold, and steryl esters two- to sixty-fold during incubation. In contrast, impure cholesterol preparations had repressor activity which could be attributed to various autoxidation products of cholesterol which accumulate with time (*11*). Among many oxysterols tested, the greatest repressor activity is produced by hydroxyl or keto substituents at C-25, C-26 or C-15 (half maximal repressor activity at ~10^{-8} M), while substitution at various other sites produces a range of less active compounds. In addition, both the 3ß-hydroxyl group and the full length side chain of cholesterol are required for high activity and the stereochemistry of the second oxygen function and of the sterol ring system also govern repressor potency (*1, 12*).

Subsequent investigations have focused mainly on one of the most potent oxysterols discovered, 25-hydroxycholesterol (*13*). It has been established that: **1.** At low concentrations, 25-hydroxycholesterol and other oxysterols specifically repress cholesterol biosynthesis without affecting the rates of acetate incorporation into CO_2 and fatty acids, or the rates of protein, RNA and DNA synthesis (*11, 13*). Moreover, supplementation with cholesterol rescues cells from growth arrest by oxysterols (*14*). **2.** 25-Hydroxycholesterol represses the mRNA level for several cholesterogenic enzymes, HMGR (*15*), HMG CoA synthase (*16*) and farnesol pyrophosphate synthetase (*17*), as well as the low density lipoprotein (LDL) receptor (*18*). **3.** The promoter regions of the genes for each of these proteins contain one or more copies of an octameric sequence termed the sterol regulatory element (*19, 20*). Experiments with chimeric gene constructs indicate that this octamer may be a transcriptional enhancer for the synthase and LDL receptor genes whose function is repressed by 25-hydroxycholesterol (*19*). Recent work indicates that the role of this sequence may be somewhat different in the HMGR promoter (*21*). **4.** Among the regulated cholesterogenic enzymes, the activity of HMGR falls the most rapidly after addition of oxysterols, with a half-life of ~1 h (*13*). This decline in activity is due in part to an increase in the rate of degradation of HMGR enzyme protein (*6, 7*) as well as the repression of gene transcription.

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The Oxysterol Hypothesis

The striking potency and the regulatory and structural specificity of oxysterol action, and the lack of effect of pure cholesterol, led to the hypothesis that these compounds were mimicking the action of endogenous oxysterol derivatives which are the natural feedback regulators of the cholesterol biosynthetic pathway (*1*). The model, shown in Figure 1, illustrates two points. One is that a number of repressor oxysterols (shown enclosed in boxes) are produced normally as biosynthetic intermediates or products of cholesterol metabolism and could function as signal molecules. This suggests that oxysterol regulation could be a flexible system, able to respond to the diverse metabolic roles cholesterol serves in different organs. Secondly, the model accounts for the structural specificity of oxysterol activity and the specific effect on cholesterogenic gene expression and degradation of HMGR by proposing that a regulatory protein (receptor) must exist in cells to mediate the regulatory response.

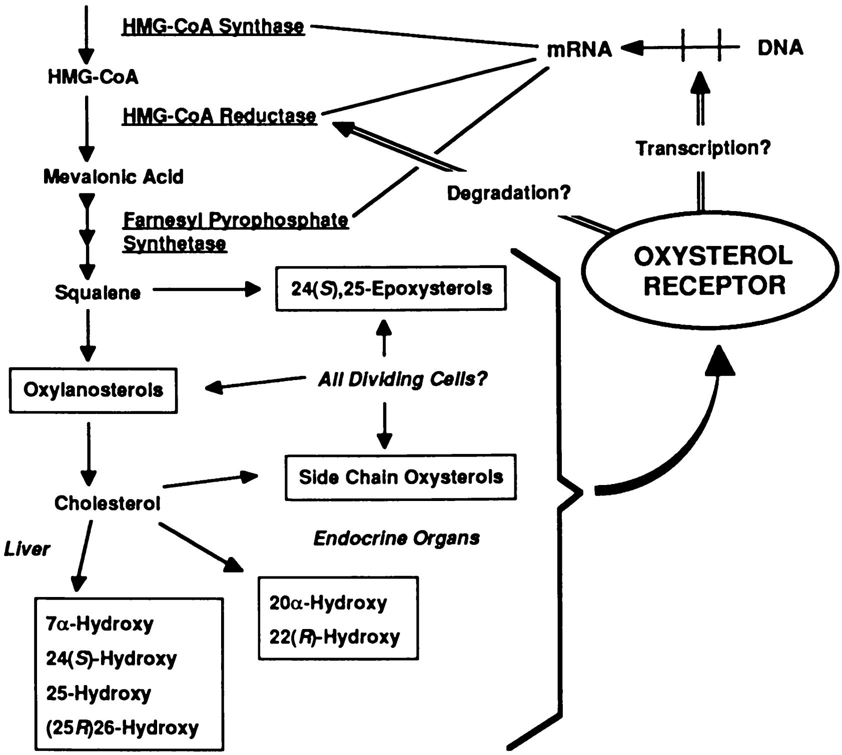
HMG-CoA Synthase
HMG-CoA
HMG-CoA Reductase
Mevalonic Acid
Farnesyl Pyrophosphate Synthetase
Squalene
24(S),25-Epoxysterols
All Dividing Cells?
Oxylanosterols
Side Chain Oxysterols
Cholesterol
Liver
Endocrine Organs
7α-Hydroxy
24(S)-Hydroxy
25-Hydroxy
(25R)26-Hydroxy
20α-Hydroxy
22(R)-Hydroxy
mRNA
DNA
Transcription?
Degradation?
OXYSTEROL RECEPTOR

Figure 1. Oxysterol regulatory model.

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Elements of the Model—The Oxysterol Receptor

Discovery. As predicted by the model, a protein with high affinity for 25-hydroxycholesterol, referred to as the oxysterol binding protein or receptor, was identified in the cytosolic fraction of cells (*22, 23, 24*). Strong support for the involvement of the receptor in the regulatory response comes from the linear correlation of the relative binding affinities of 62 oxysterols for the oxysterol receptor *in vitro* with their activities as repressors of HMGR enzyme activity in cultured cells (*12, 25, 26, 27, 28*). Furthermore, the kinetics of association and dissociation of 25-hydroxycholesterol with the receptor in intact cells is commensurate with the timing of the of the cellular response (*23, 29*). An oxysterol receptor has been found in the cytosolic fraction of all mammalian cells tested, including cultured cell lines and various mouse, rat, hamster and human tissues (*24, 30, 31, 32*), and this correlates with the general sensitivity of mammalian cells to oxysterol regulation. No evidence has been obtained so far for the presence of other proteins with high affinity for oxysterols during the fractionation procedures used to purify the receptor or in investigations of binding proteins in nuclear extracts.

Structural Characterization. We have used partially purified oxysterol receptor preparations from mouse fibroblast L cell cultures to study the specificity and kinetics of oxysterol binding and to develop a subunit model for the protein based on changes in its hydrodynamic properties under different conditions (*12, 33*). An oxysterol photoaffinity label was produced to aid in purification and identification of the receptor on denaturing gels (*25*). The protein has been purified by Dawson *et al* (*32*) from hamster liver and in our laboratory from mouse L cells (*34*).

The receptor-oxysterol complex has a sedimentation coefficient of 7.5S and an apparent molecular weight of 169,000. This form was presumed to be a dimer since it could be reversibly dissociated to a 4.2S, 95 kDa sterol-binding form in the presence of 2.5 M urea at acid pH (*33*). The 4.2S form exhibits greatly increased rates of oxysterol association and dissociation although the Kd for 25-hydroxycholesterol is nearly the same as for the 169 kDa form. Analysis of the purified receptor by SDS-PAGE showed one major protein band of 95 kDa along with several very minor bands of lower molecular weight. A specific, covalently-bound ^{3}H-photoaffinity ligand was associated almost exclusively with the 95 kDa band in agreement with the subunit molecular weight estimated by the hydrodynamic measurements (*25, 34*). These results are consistent with the liganded receptor being a dimer composed of two identical sterol-binding subunits or of two nonidentical subunits of similar M_r.

There is also evidence from sucrose density gradients and gel filtration chromatography that the unliganded receptor is larger than the liganded form (*33*). The unliganded receptor had a calculated molecular weight of 236,000 which suggests that a 67,000 subunit dissociates when an oxysterol ligand is bound. Although the physiological relevance of the larger molecular weight form of the receptor is not clear, it suggests the possibility that the receptor associates with other specific proteins in the cell which may participate in the mechanism of transcription regulation. The unliganded receptor is difficult to purify and analyze since it is very unstable.

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Irreversible conversion of the 7.5S form of the receptor to a ~4S form by a factor(s) present in the unfractionated cytosol of L cells, and failure of a spectrum of protease inhibitors to prevent this conversion, was noted in early studies of the receptor (*33*). SDS-PAGE of the ~4S photoaffinity-labeled form from L cells showed that irreversible conversion by the cytosolic factor(s) was associated with the production of smaller M_r polypeptides, the principal ones having M_r values of about 30,000 and 55,000 (*34*). This observation suggests that a specific region of the receptor structure is particularly susceptible to the action of proteases. To investigate this further we characterized a receptor form produced by limited digestion with trypsin, chymotrypsin and endoproteinase GluC (*35*). Treatment with each of these proteases converts the receptor to a 44 kDa form based on hydrodynamic measurements. SDS-PAGE of photoaffinity labeled preparations indicates that the oxysterol binding site is on a 28 kDa fragment within the 44 kDa limit form of the receptor. The limit proteolytic form exhibits the high affinity and structural specificity for oxysterols of the native dimeric receptor with an increase in the rate of association with 25-hydroxycholesterol.

Receptor Sequence. Dawson *et al* (*36*) isolated and sequenced cDNA clones for the rabbit receptor, and the sequence of the very similar human receptor has also been reported (*37*). The rabbit sequence predicts a protein of 809 amino acids with a M_r of ~90,000. The first 100 amino acids of the sequence are rich in glycine, alanine and proline, possibly similar to a short region in the *gap* protein, but the significance of this is not clear. The only other structural feature apparent in the receptor sequence is a possible leucine zipper motif. This type of structural motif participates in the dimerization of some transcription factors (*38*). Dawson *et al* (*36*) speculated that this sequence in the receptor might be involved in heterodimer formation with a transcription factor that regulates cholesterogenic genes and that heterodimer formation would be further stabilized by the association of a region of acidic amino acids on the receptor with basic amino acids on the transcription factor.

The most significant aspect of the receptor sequence is its lack of any other obvious homology to proteins in sequence data bases, most notably to the family of DNA-binding transcription factors which include the steroid hormone receptors. We had originally proposed that the protein might be a member of this family because it binds a small effector ligand which regulates transcription and because it initially seemed to have similar subunit properties to the glucocorticoid receptor. If homologies can eventually be established between the oxysterol receptor and other proteins it may perhaps speak to the receptor's role in the regulation of cholesterol biosynthesis and suggest a mechanism of action. cDNA clones also provide the opportunity to create receptor-deficient cell lines by various molecular techniques for a direct test of receptor function.

Mechanism of Action. DNA Binding. Since the most straightforward model for the receptor's mechanism of action would be control of transcription by direct binding to promoter sequences, it was of importance to investigate the receptor's affinity for DNA. Low affinity binding to nonspecific DNA is a property of many transcription factors and therefore we began by defining the receptor's affinity for non-specific calf

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thymus DNA bound to cellulose. Two conditions had to be met to obtain binding of the receptor-oxysterol ligand complex to DNA-cellulose: contaminating RNA in receptor preparations had to be removed by either ribonuclease A treatment or anion exchange chromatography and, because of the sensitivity of binding to ionic strength, salts had to be removed by dialysis or desalting chromatography. Optimum binding occurred at 15 mM KCl and binding was prevented by ~75 mM KCl at pH 7. $ZnCl_2$ and $CoCl_2$ promoted receptor binding, while $MgCl_2$, $MnCl_2$, and $CaCl_2$ had no effect. Binding was strongly influenced by pH: at pH 5.5 a concentration of >200 mM KCl was required to dissociate the receptor-DNA complex. The increased binding at acid pH may suggest that the monomeric receptor binds to DNA with higher affinity than the dimer, since reversible dissociation appears to be favored at a pH less than 6. However, clearly demonstrable, reversible dissociation to the native monomeric form has been achieved only in the presence of high salt and urea, conditions that do not allow measurements of affinity for DNA. We have used sterol-binding fragments of the receptor generated by limited proteolysis as an approach to this issue and find that binding of the proteolytic limit form of the receptor to DNA cellulose is stable to higher salt concentrations than binding of the intact receptor (*35*).

We used the results of the above studies as a guide to investigate the affinity of the receptor for specific regulatory DNA sequences. A plasmid containing a 510 bp fragment of the HMGR promoter was obtained from Dr. Kenneth Luskey, University of Texas Health Science Center at Dallas, and the full length fragment as well as smaller segments were prepared. We also used a 42 bp synthetic oligonucleotide corresponding to the sterol regulatory element of the LDL receptor promoter and single and double strand 30 base synthetic oligonucleotides corresponding to the oxysterol regulatory element of the HMGR promoter. Binding to the oxysterol receptor was tested by the conventional polyacrylamide gel DNA binding assay (gel retardation assay). Shifted bands corresponding to protein-DNA interactions were seen for some fragments using impure preparations of the oxysterol receptor, but this activity failed to copurify with the receptor. In addition, although the shifted bands were formed in the presence of a 6000-fold excess of competing poly[d(IC)] DNA, small molecular weight fragments of nonspecific DNA seemed to compete for protein binding on an equal molar basis with specific DNA. Binding studies carried out in the presence of $ZnCl_2$ or at pH 5.5 or with the proteolytic limit form of the receptor also failed to provide convincing evidence for specific binding. Binding studies with high-salt nuclear extracts showed several shifted bands, especially when one of the single strand 30 oligomers from the HMGR promoter was used as described (*39*). However, purified oxysterol receptor fractions had no effect on this banding pattern when added to the nuclear protein incubations.

Cellular Localization. Another test of the hypothesis that the oxysterol receptor is a transcription factor was to determine if the receptor is present in the nuclei of cells. We utilized a cell enucleation technique (*40, 41*) to determine the receptor's locale within the intact cell. L cells were incubated with cytochalasin B and ^{3}H-25-hydroxycholesterol (to label the oxysterol receptor) or ^{3}H-triamcinalone acetonide (to label the glucocorticoid receptor). The cells were then separated into nucleoplast and cytoplast fractions on Percoll gradients. Assays for DNA (nuclei) and lactate dehydrogenase (cytoplasm) demonstrated that enucleation of the cells was achieved. Sucrose density gradient centrifugation demonstrated that the steroid-bound glucocorticoid receptor was found predominantly in the nucleus, as expected, while the pattern of recovery of the oxysterol receptor was consistent with a cytosolic location. The cytoplasmic location of the oxysterol receptor by this technique is consistent with other cell fractionation experiments we have carried out and may indicate that the receptor does not act directly as a nuclear transcription factor.

Oxysterol Resistant Cell Lines. Another means of investigating the oxysterol regulatory system is to select cell lines resistant to growth-inhibition by oxysterols in the absence of a compensating supply of cholesterol. Since oxysterols repress several enzymes of the cholesterol biosynthetic pathway, such mutants may be defective in a general factor that regulates cholesterogenic gene transcription. Several groups of investigators have reported the isolation of Chinese hamster ovary or lung cell lines resistant to 25-hydroxycholesterol and have shown that the levels of cholesterogenic enzymes in these lines are not repressed by oxysterol treatment (*42, 43, 44, 45, 46, 47, 48*). We found that the oxysterol-resistant cell lines selected in this laboratory appear to have a normal oxysterol receptor structure as monitored by sucrose density gradient analysis and by immunostaining of SDS-PAGE Western blots (unpublished). Therefore, this avenue of research has not yet provided proof of the role of the receptor protein in this regulatory response.

Conclusions. Strong evidence for the involvement of the oxysterol receptor in the regulation of cholesterol biosynthesis is provided by the correlation of its binding affinities for various oxysterols with their activities as repressors of HMGR activity (*12, 25, 26, 27, 28*), as well as the presence of the receptor in all cell types and the apparent absence of any other oxysterol binding activity in cells. However, the biochemical information obtained so far and the sequence of the receptor have not revealed the mechanism for oxysterol regulation of cholesterogenic gene transcription. This situation is not uncommon in research on signal receptors in which the initial characterization of activity is based on ligand binding affinity, or in cases where proteins are initially characterized by sequence data alone. The evidence that the receptor is predominantly a cytosolic protein and does not appear to have specific affinity for the sterol regulatory DNA sequence element cannot be taken as conclusive that the receptor is not a transcription factor, but it does suggest that it may regulate cholesterol biosynthesis by a less direct mechanism. These might include protein-protein interactions or enzymatic modification of other transcription factors. It could also be proposed that the receptor does not participate in transcriptional regulation and may only be involved in controlling the rate of HMGR protein degradation. This is being tested directly by correlating the binding affinity for oxysterols with the separate regulatory activities.

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Elements of the Model—Endogenous Regulatory Oxysterols.

The other critical requisite of the oxysterol hypothesis is to identify endogenous regulatory oxysterols and to discover how their levels are controlled in cells. A number of active repressor oxysterols are produced normally as biosynthetic intermediates or products of cholesterol metabolism. All cells synthesizing cholesterol produce the obligate precursors, 32-hydroxylanosterol and 32-oxolanosterol, and 24(S),25-epoxycholesterol can be formed by a branch pathway starting from squalene 2,3(S);22(S),23-dioxide. Derivatives of cholesterol hydroxylated in the 7α and 26-positions are produced in liver during bile acid production, and side chain hydroxylations in the 20α- and 22R-positions are the first steps in the conversion of cholesterol to steroid hormones in endocrine organs. In addition, the 7β-, 24(S)-, and 25-hydroxy and 7-keto derivatives of cholesterol have been isolated from various cells and tissues.

It must be emphasized that autoxidation artifacts are a serious concern during analysis; oxysterols are usually present only at .001 to .005% of the concentration of cholesterol in cell extracts. Extraction procedures must be conducted in the absence of oxygen (*49*) or in the presence of antioxidants, and the monohydroxylated sterols separated as rapidly as possible from the oxysterol fraction. For certain sterols such as 7α-, 24(S)- and (25R)26-hydroxycholesterol, isolation of only one of two epimers can indicate an enzymatic origin. The presence of other oxysterols such as 7β-hydroxycholesterol and 7-ketocholesterol are indications of probable autoxidation problems.

Oxysterols in Cultured Cells. A thorough survey of the regulatory sterols in cultured cells and mouse liver has been conducted using the repression of HMGR in mouse fibroblasts as a bioassay (*50, 51, 52*). This unique, comprehensive effort was required to insure that no unanticipated regulatory compounds were missed. Extraordinary care was taken to monitor for and suppress the production of autoxidation artifacts. The presence of 24(S),25-epoxycholesterol and 25-hydroxycholesterol was demonstrated in Chinese hamster lung cells grown in delipidated serum (*52*). The level of these sterols was in the range required for weak repression of HMGR. Addition to the culture medium of a concentration of mevalonic acid high enough to repress the HMGR by 90% resulted in the appearance of two new regulatory oxysterols, 32-hydroxylanosterol and 32-oxolanosterol, such that the total repressor activity in the cell extracts increased by 30-70% (*51*). Mevalonic acid also increased a number of other sterol biosynthetic intermediates such as lanosterol, desmosterol and 7-dehydrocholesterol, but caused no change in the concentration of cholesterol, 24(S),25-epoxycholesterol and 25-hydroxycholesterol (*51*). These data indicate that oxysterols regulate the level of HMGR in dividing cell cultures; however, the large stimulation of isoprenoid production by an influx of mevalonic acid may not be related to any normal cellular condition.

Several other methods have been described for repressing cholesterol synthesis in cultured cells by manipulating intracellular sterol levels. One method is to supplement the medium with LDL. This has been interpreted to demonstrate that cholesterol, when delivered by endocytosis through the lysosomal compartment, can produce a

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regulatory effect (*53*). Confounding the use of LDL is the evidence that even freshly isolated LDL contains oxysterol compounds in concentrations which may be sufficient to repress cholesterol biosynthesis (*49, 54, 55, 56*). No attempt to utilize reconstituted, oxysterol-free LDL has been reported. A second method of repressing cholesterol synthesis is based on the observation that hydrolysis of sphingomyelin in the plasma membrane of cells results in the internalization of plasma membrane cholesterol (*57*). HMGR activity is repressed as a consequence of this cholesterol influx and the levels of biosynthetically labeled "polar sterols" in the cells rise (*58*). These oxysterols have not been identified. A third method of repressing sterol synthesis is by partial inhibition of 2,3-oxidosqualene cyclase (*59*) or lanosterol 14-demethylase (*60*) enzymes by drug treatment. This increases the concentrations of 24(S),25-oxidocholesterol and 32-oxylanosterol derivatives, respectively, with a concomitant reduction in HMGR activity. Although these results support the oxysterol hypothesis, they do not prove that these particular oxysterol intermediates regulate cholesterol biosynthesis under normal circumstances.

Oxysterols in Liver and Other Tissues. The liver plays a critical role in cholesterol homeostasis and it is of central importance that hepatic oxysterols be identified and their abundance related to the level of HMGR activity. Saucier et al (*50*) found low levels of six oxysterols, 7α-, 7ß-, 24(S)-, 25- and (25R)26-hydroxycholesterol and 7-ketocholesterol in the livers of mice fed cholesterol-free diets (see Table I). The concentration of 24(S)-hydroxycholesterol in the free sterol fraction increased 3 and 5-fold in the livers of mice fed purified, oxysterol-free cholesterol for one and two nights, respectively, and the amount of 24(S)-hydroxycholesterol in the fatty acid ester fraction increased even more dramatically. An increase in the concentration of 25-hydroxycholesterol was found after one and two nights, and after two nights an in-

Table I. Side Chain Oxygenated Sterols in Mouse Liver (ng/g liver)

Cholesterol derivative	Control Free (Esters)	Cholesterol fed Free (Esters)	Fed/Control Total
24(S)-hydroxy			
1 night	220 (510)	650 (2960)	5
2 nights	180 (140)	900 (3700)	14
25-hydroxy			
1 night	3 (40)	70 (20)	2.1
2 nights	nd (nd)	55 (70)	—
(25R)26-hydroxy			
1 night	90 (310)	140 (160)	0.75
2 nights	40 (130)	220 (220)	2.6

nd - Not detected. (Limit of detection is ~ 2 ng/g liver.)
SOURCE: Adapted from ref. 50.

crease was found for 26-hydroxycholesterol. The concentrations of the other identified oxysterols did not change after cholesterol feeding. Other oxysterols which have been suggested as candidate regulators including 24(S),25-epoxycholesterol and the 32-hydroxy and 32-aldehyde derivatives of lanosterol were not found in this study. The increase in 24(S)-hydroxycholesterol is especially intriguing since this sterol is a potent repressor of HMGR activity, it is not known to be a normal intermediate in the metabolism of cholesterol to bile acids and no direct autoxidative pathway leading to this sterol from cholesterol has been discovered (*61*). 24(S)-Hydroxycholesterol has also been found in brain (*61, 62*) and adrenal glands (*63*), two organs which exhibit large regulatory fluctuations in HMGR activity.

Other investigators have suggested that 26-hydroxycholesterol may be a regulator of oxysterol biosynthesis (*64, 65, 66, 67*). This oxysterol is a normal intermediate in a minor pathway of bile acid synthesis and it is a potent repressor of HMGR in cell cultures (*12, 65*). The cDNA for the mitochondrial cholesterol C26 hydroxylase has been cloned and the message for the gene is expressed in many cell types (*64*). One argument for a regulatory role for 26-hydroxycholesterol is that cerebrotendinous xanthomatosis patients, which have a deficiency in the 26-hydroxylase enzyme, develop xanthomas consisting of large deposits of cholesterol and cholestanol in many tissues. Furthermore, a 30 to 100 percent increase in whole body cholesterol biosynthesis was demonstrated in two patients compared to normal controls (*68*). Although this is taken as evidence that 26-hydroxycholesterol normally feedback regulates cholesterol biosynthesis, supplementation of the cerebrotendinous xanthomatosis patients' diet with the bile acid, chenodeoxycholic acid, appears to completely relieve the development of the disease and its accompanying sterol deposits, despite the presumably continued lack of 26-hydroxycholesterol in tissues (*69*). The increase in cholesterol synthesis in the untreated patients may be due to a lack of normal bile acid production and the consequent reduction in cholesterol reabsorption.

Conclusions. It will be important to quantitate 24(S)- and (25R)26-hydroxycholesterol and other oxysterols in a variety of animal species, cell lines and tissues and to correlate their levels with the repression of HMGR as a function of the amount and duration of cholesterol feeding. Once endogenous regulatory sterols are clearly identified, investigation of how their concentrations are regulated in cells can begin. For example, if hydroxylation of cholesterol is determined by substrate availability in the mitochondria the situation would be reminiscent of the regulation of steroid hormone production. In that pathway the activity of the C22 and C24 side-chain hydroxylating enzymes is regulated by the movement of cholesterol to the mitochondrial inner membrane, and by analogy cholesterol biosynthesis would be regulated by proteins which control cholesterol movement to the site of side chain hydroxylation. On the other hand, if evidence is obtained that enzyme activity increases after cholesterol feeding the mechanism of control would have to be sought in the transcriptional or post-transcriptional control of enzyme activity.

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Chapter 9

Dual-Action Inhibitors of Cholesterol Biosynthesis

Lanosterol Analogs That Inhibit Lanosterol 14α-Methyl Demethylase and Suppress 3-Hydroxy-3-methylglutaryl–Coenzyme A Reductase Activity

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Lanosterol 14α-methyl demethylase (P-450$_{DM}$) is the cytochrome P-450 monooxygenase which oxidatively removes the 14α-methyl group of lanosterol. This demethylation is the rate limiting step in the conversion of lanosterol to cholesterol. The intermediates in this transformation are known to bind very tightly to P-450$_{DM}$ and have been implicated in the regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) activity, the rate limiting enzyme in overall cholesterol biosynthesis. Lanosterol analogs **32a**, **32b**, and **33**, which are methylated analogs of the intermediates generated during the removal of the 14α-methyl group by P-450$_{DM}$, have been prepared and their biological activities assessed. All three compounds were found to inhibit P-450$_{DM}$ and to cause the suppression of HMGR activity. Studies with a P-450$_{DM}$-deficient mutant suggest that the mechanism of suppression of HMGR by these compounds may be the competitive inhibiton of P-450$_{DM}$ causing the build-up of the natural intermediates generated during the removal of the 14α-methyl group which in turn suppress HMGR activity.

Early epidemiological studies suggested a graded relationship between serum cholesterol levels and the risk of coronary heart disease (CHD) which is a major medical problem in the US *(1)*. More recently, the results of numerous clinical studies have indicated that the lowering serum cholesterol levels may indeed reduce the risk of CHD and even foster the regression of atherosclerotic lesions *(2-4)*. Serum cholesterol levels can often be controlled by restricting their dietary intake of cholesterol; however, with a significant number of patients, this approach does not result in the reduction of serum cholesterol concentrations to beneficial levels. In these patients, it is imperative that other methods for the control of serum cholesterol levels be available. A potential approach to the treatment of hypercholesterolemia in these patients is the development of inhibitors of cholesterol biosynthesis.

Background

Regulation of HMGR Activity. The rate-limiting enzyme of cholesterol biosynthesis is 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, EC

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1.1.1.34). HMGR catalyzes the reductive deacylation of 3-hydroxy-3-methylglutaryl coenzyme A **1** (HMG-CoA) to yield mevalonate **2** (Scheme 1) *(5-7)*. It has been suggested that in cultured mammalian cells the activity of HMGR is regulated through a multivalent feedback mechanism mediated by end product sterols along with an additional metabolite of mevalonate **2** *(8-10)*. Addition of highly purified cholesterol to cultured cells does not affect the activity of HMGR or the rate of sterol synthesis; however, numerous oxygenated sterols have been shown to be potent suppressors of HMGR activity resulting in marked reductions of cholesterol production *(11-13)*. These observations led Kandutsch and Chen *(14)* to hypothesize that oxysterols, rather than cholesterol, may function as the natural regulators of HMGR activity and sterol synthesis. The structures of representative oxysterols known to suppress HMGR activity are depicted in Figure 1.

The oxysterol used most frequently in the study of HMGR regulation is 25-hydroxycholesterol **3** (Figure 1). This oxysterol is a potent inhibitor of HMGR activity (IC_{50}<1 μM) *(11-14)*. Repression of the synthesis of immunoprecipitatable HMGR by oxysterol **3** has been demonstrated by Faust's group *(15)* in a CHO-derived compactin-resistant cell line that over produces HMGR (UT-1) and by Sinensky *et al. (16)* in Chinese hamster ovary (CHO) cells. Luskey *et al.* have shown that the level of mRNA for HMGR in UT-1 cells is reduced in the presence of oxysterol **3** *(17)*. Similar results were obtained by Leonard, Chen, and Taylor in wild type CHO cells *(11-13)*. With these facts in mind, Kandutsch and Thompson *(18)* proposed the following model for the regulation of HMGR activity by oxysterols. The oxysterol binds to a cellular protein and the resultant oxysterol-protein complex then acts to repress transcription of the gene for HMGR. In support of this model, Kandutsch *et al.* have isolated a protein which exhibits high affinity and low capacity for 25-hydroxycholesterol **3** and other oxysterols which are known to suppress HMGR activity *(18-21)*. Although 25-hydroxycholesterol has been isolated from cells, there is no direct evidence that this oxysterol functions as the natural regulator of cholesterol biosynthesis *(22)*.

A number of the oxysterols that are potent suppressors of HMGR activity are natural catabolites of cholesterol *(21)*. For example, 7α-hydroxycholesterol **4** and 26-hydroxycholesterol **5** are intermediates in the formation of bile acids from cholesterol and 20(R)- and 22(S)-hydroxycholesterol **6** & **7** are produced during the cleavage of the side chain of cholesterol in the formation of pregnanes. However, cells which do not catabolize cholesterol also regulate the activity of HMGR sterol synthesis, which precedes DNA synthesis; this process can be inhibited by known oxysterol repressors of HMGR *(23)*. These facts suggest that an oxysterol or some combination of oxysterols which are precursors to cholesterol may be important in the regulation of cholesterol biosynthesis.

In the biosynthesis of cholesterol, oxygenated sterols are generated during the oxidative removal of the three "extra" methyl groups (C-30, C-31, C-32) of lanosterol *(5-7)*. 32-Oxygenated lanosterol analogs (compounds **8** through **11**) have been shown to decrease the activity of HMGR in Chinese hamster lung cells *(23)* and compounds **8** and **9** have been shown to bind to the oxysterol binding protein *(21)*. 32-Oxylanosterols are formed during the removal of the 14α-methyl group of lanosterol **18** by lanosterol 14α-methyl demethylase (P-450_{DM}), a membrane-bound cytochrome P-450 monooxygenase *(24)*. The accumulation of these 32-oxylanosterols has been shown to be inversely correlated with the activity of HMGR *(25,26)* and they have been shown to be effective inhibitors of HMGR activity when added to cultured cells *(23)*.

Trzaskos *et al.* have shown that utilization of conditions expected to effect single catalytic turnover of P-450_{DM} results in the build-up of the intermediate oxysterols **8** and **10** *(27)*. In addition, competitive inhibition of P-450_{DM} by lanosterol **18** or dihydrolanosterol **23** or the loading of the microsomes with cholesterol causes the accumulation of 32-oxysterols **8** and **10** *(27)*. The azole

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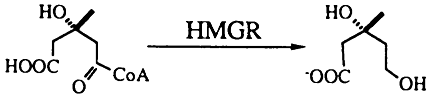
HO
HOOC
O
CoA
HMGR
HO
-OOC
OH

1 HMG-CoA 2 Mevalonate

Scheme 1

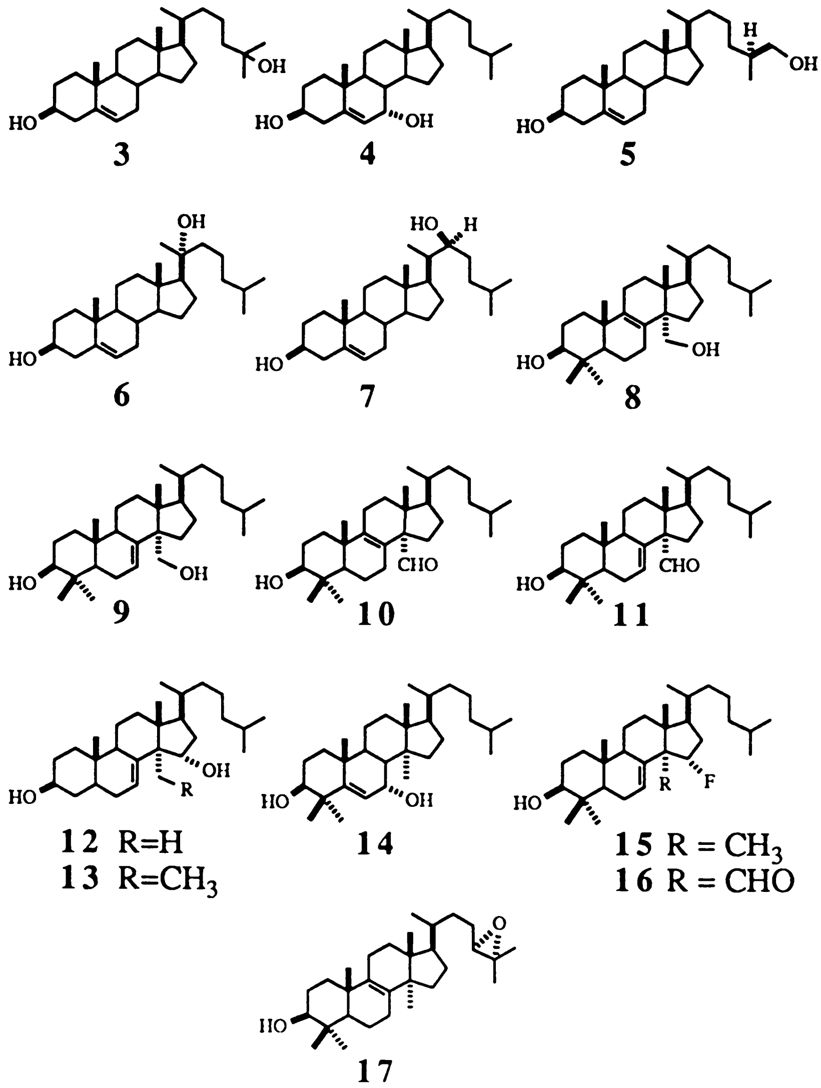
OH
HO
3
OH
HO
4
H
OH
HO
5
OH
HO
6
HO H
HO
7
OH
HO
8
OH
HO
9
CHO
HO
10
CHO
HO
11
OH
R
HO
12 R=H
13 R=CH3
OH
HO
14
R
F
HO
15 R = CH3
16 R = CHO
O
HO
17

Figure 1. Known oxysterol suppressors of HMGR activity.

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inhibitors of P-450$_{DM}$, miconazole and ketoconazole, also promote the build-up of these 32-oxysterols in a dose dependent manner. Under all of the above conditions, aldehyde **10** and alcohol **8** were found to accumulate in a ratio of 3:1, respectively *(25,28)*.

The studies with miconazole are of particular interest since miconazole causes a biphasic modulation of HMGR which correlates well with the build up of 3β-hydroxylanost-8-en-32-al **10**. Treatment of the P-450$_{DM}$-deficient mutant AR45 with aldehyde **10** causes a reduction of HMGR activity due to a decrease in the immunoreactive HMGR protein; this result clearly illustrates that metabolic activation of this aldehyde by P-450$_{DM}$ is not required for it to cause suppression of HMGR activity *(25,28)*. These results lend strong support to the hypothesis that 3β-hydroxylanost-8-en-32-al **10** is an endogenously generated regulator of HMGR activity.

In contrast to oxycholesterols, the mechanism of action of oxylanosterols has not been thoroughly characterized, but there are indications that these compounds may regulate HMGR by post-transcriptional mechanisms. Translational regulation of HMGR gene expression was first proposed by Peffley and Sinensky in 1985 *(29)*. Addition of 25-hydroxycholesterol **3** to a mevalonate auxotroph cell line resulted in a decrease in HMGR mRNA and an increase in the degradation rate of HMGR protein. Treatment of cells with both 25-hydroxycholesterol **3** and mevalonate **2** yielded a further reduction in enzyme activity without any additional change in mRNA levels or HMGR degradation rate. Nakanishi *et al. (30)* reported similar results using CHO cells incubated acutely with high levels of compactin, a competitive inhibitor of HMGR. In this system, addition of mevalonate **2** to 25-hydroxycholesterol **3** treated cells results in an 80% decrease in mRNA translation and a 5-fold increase in the degradation of HMGR. The conclusion drawn by both of these groups was that post-transcriptional regulation of HMGR requires a non-steroidal mevalonate metabolite. However, these results are also consistent with the second inhibitor being an oxysterol which acts by a different mechanism than 25-hydroxycholesterol **3**.

Recent evidence suggests that not all oxysterols act through a common mechanism. Treatment of cultured mammalian cells with the lanosterol analog, 15α-fluorolanost-7-en-3β-ol **15**, a competitive inhibitor of P-450$_{DM}$, results in the accumulation of 15α-fluoro-3β-hydroxylanost-7-en-32-al **16** accompanied by a decrease in HMGR activity and immunoreactive protein. Interestingly, an increase in HMGR mRNA was also observed *(31)*. Despite this increase in HMGR mRNA in cells treated with 15α-fluoro compound **15**, the rate of synthesis of HMGR protein is decreased while the degradation rate of the enzyme is unaffected (unpublished results, D. A. Leonard). Additional evidence that some oxylanosterols inhibit HMGR by post-transcriptional mechanisms was recently presented by Ko and coworkers *(32)*. Their preliminary characterization of a number of different 14,15-substituted oxylanosterols showed that in contrast to 25-hydroxycholesterol **3**, some of these compounds inhibit enzyme activity without decreasing mRNA levels and stimulate, rather that inhibit, LDL receptor expression.

Oxylanosterols with an oxygen in the side chain have also been reported to inhibit HMGR post-transcriptionally *(33)*. Addition of 24(S),25-oxidolanost-8-en-3β-ol **17** to cultured rat hepatocytes causes a six-fold decrease in HMGR synthesis and a two-fold increase in enzyme degradation without a change in the level of HMGR mRNA. Although both mevalonate **2** and 25-hydroxycholesterol **3** are required for maximal inhibition of HMGR synthesis in fibroblasts, addition of mevalonate to a mevalonate auxotroph cell line treated with 24(S),25-oxidolanost-8-en-3β-ol **17** does not result in any further inhibition of HMGR synthesis *(33)*. This suggests that 24(S),25-oxidolanost-8-en-3β-ol **17** and mevalonate **2** are inhibiting HMGR synthesis by a common post-transcriptional mechanism.

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Mechanism of Removal of the 14α-Methyl Group of Lanosterol by P-450$_{DM}$. As mentioned earlier, P-450$_{DM}$ catalyzes the oxidative removal of the 14α-methyl group of lanosterol *(24)*. This constitutes the first step in the conversion of lanosterol **18** to cholesterol and is the rate limiting enzyme in this process. P-450$_{DM}$ has been purified from both yeast *(34)* and mammalian (rat liver) sources *(35,36)*, and the yeast gene has been isolated *(37,38)*. The proposed mechanism of the demethylation catalyzed by P-450$_{DM}$ is depicted in Scheme 2. The 14α-methyl group of lanosterol **18** is first hydroxylated to give the hydroxymethyl intermediate **19** followed by oxidation to the corresponding aldehyde **21** presumably *via* the geminal diol **20**; oxidative elimination of C-32 as formic acid then yields triene **22**. Each of the three steps is NADPH and O_2 dependent. In addition, functional reconstituted systems from both rat liver and yeast require NADPH-cytochrome P-450 reductase. Studies with purified enzyme systems suggest that all of the oxidations are catalyzed by a single cytochrome P-450 *(35,36,39)*. As expected for a cytochrome P-450 monooxygenase system, the oxidative removal of C-32 is carbon monoxide sensitive *(40-43)*.

Although lanosterol **18** is the natural substrate for demethylation, both yeast and hepatic P-450$_{DM}$ will utilize 24,25-dihydrolanosterol **23** *(27,44)*. Trzaskos and coworkers have found the K_m of lanosterol **18** in rat liver system to be 165 μM (V_{max} = 3390 pmol/min/mg) while that of 24,25-dihydrolanosterol **23** to be 312 μM (V_{max} = 645 pmol/min/mg) *(27)*. However, we have obtained a K_m of 23-33 μM for 24,25-dihydrolanosterol **23** with rat liver microsomes *(45)* which is in agreement with that found by Bossard (K_m = 32.5 μM, V_{max} = 178 nmol/mg/hr) with the purified enzyme *(36)*. In a reconstituted yeast system, the apparent K_m of 24,25-dihydrolanosterol **23** is 19.4 μM *(46)*.

The effect of the nuclear double bond position has been examined by Mitropoulos *et al.* In rat liver microsomal preparations lanost-7-en-3β-ol **26** (Scheme 3) exhibits approximately the same K_m as lanost-8-en-3β-ol **23**; however, the V_{max} of the Δ^8-isomer is about sevenfold higher than that of the Δ^7-isomer *(40)*. The conversion of tritiated lanost-7-en-3β-ol **26** to 4,4-dimethylcholesta-7,14-dien-3β-ol **27** and cholesterol has been verified (Scheme 3) *(40)*. The presence of a Δ^7 or Δ^8 double bond does appear to be required for demethylation since lanostan-3β-ol **28** is not converted to cholestan-3β-ol by rat liver preparations *(47)*.

In both yeast and hepatic systems, the ultimate products of demethylation of lanosterol **18** and 24,25-dihydrolanosterol **23** by P-450$_{DM}$ are formic acid and the conjugated dienes **22** and **25**, respectively. Trzaskos *et al.* have demonstrated the formation of diene **[24,25-^{3}H]25** from [24,25-^{3}H]dihydrolanosterol **[24,25-^{3}H]23** with the aid of the $\Delta^{8,14}$-sterol Δ^{14}-reductase inhibitor AY-9944 *(43)*. Aoyama and Yoshida have identified triene **22** as the product of demethylation of lanosterol **18** in the yeast system *(39,41)*. Using [32-^{3}H]lanost-7-ene-3β,32-diol **[32-^{3}H]9**, the formation of tritiated formic acid and THO has been observed *(48)* and similarly, using [32-^{14}C]lanost-8-en-3β-ol **[32-^{14}C]23**, the production of ^{14}C-formic acid has been verified *(40)*.

The first two oxidations carried out by P-450$_{DM}$ are considered to occur at C-32. Lanosterol **18** yields diol **19** followed by aldehyde **21** while 24,25-dihydrolanosterol **23** gives oxysterols **8** and **10**. Cell free systems from rat liver promote the conversion of both diol **8** and aldehyde **10** to cholesterol. By using appropriately labeled versions of lanost-7-ene-3β,32-diol **9**, Akhtar *et al.* have demonstrated the conversion of this diol to diene **27** with loss of C-32 as formic acid in the rat liver system *(48)*. Aldehyde **11** was found to be an obligatory intermediate in the conversion of diol **9** to diene **27**. Both the conversion of lanost-7-ene-3β,32-diol **9** and the subsequent conversion of aldehyde **11** to lanost-7,14-dien-3β-ol **27** exhibited a requirement for NADPH and O_2. Studies carried out by Akhtar suggest

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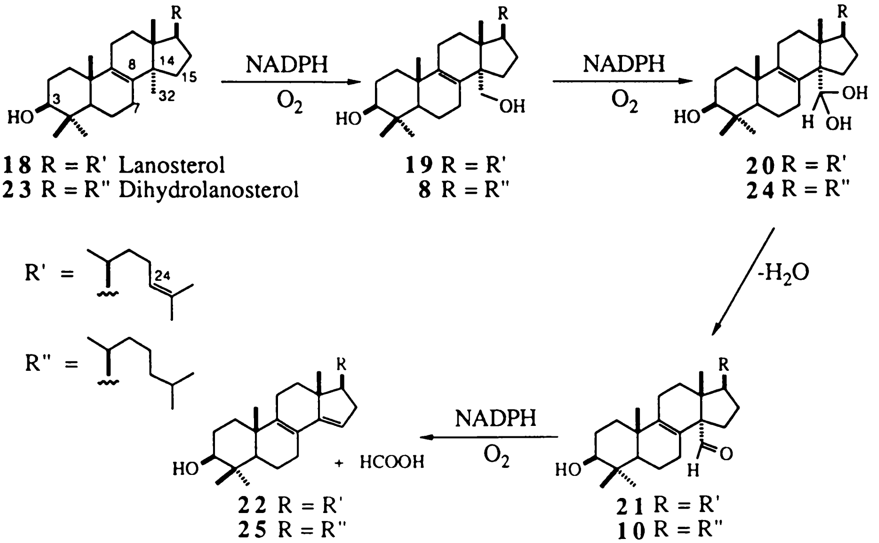
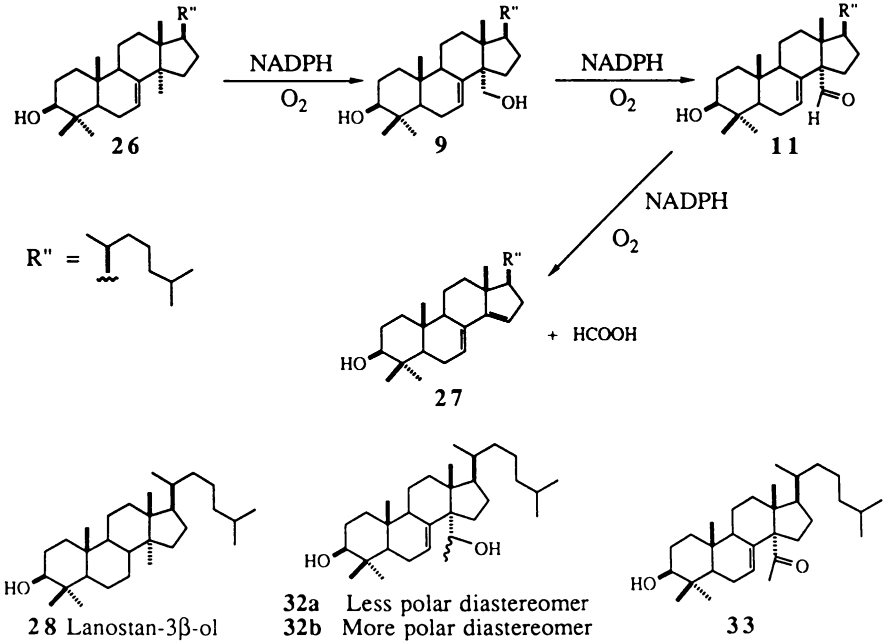

R
8
14
15
3
32
7
HO
NADPH
O2
OH
OH
H OH
18 R = R' Lanosterol
23 R = R" Dihydrolanosterol
19 R = R'
8 R = R"
20 R = R'
24 R = R"
R' =
24
R" =
-H2O
+ HCOOH
O
H
22 R = R'
25 R = R"
21 R = R'
10 R = R"

Scheme 2


R"
HO
26
NADPH
O2
OH
9
O
H
11
R" =
+ HCOOH
27
28 Lanostan-3β-ol
32a Less polar diastereomer
32b More polar diastereomer
33

Scheme 3

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that the second oxidation (*ie.* diol **9** to aldehyde **11**) may occur in a stereospecific manner *(48)*.

The natural Δ^8-diols have also been studied. Akhtar *et al.* have demonstrated the metabolism of [3,32-^{3}H]lanost-8-ene-3β,32-diol **[3,32-^{3}H]8** to [3-^{3}H]4,4-dimethylcholesta-8,14-dien-3β-ol **[3-^{3}H]25** by microsomal preparations from rat liver *(44)*. Recently, Bossard has found the K_m of lanost-8-ene-3β,32-diol **8** to be 5.1 μM (V_{max} = 688 nmol/mg/hr) in a reconstituted system from rat liver indicating a high affinity of the cytochrome for this intermediate diol (K_m of 24,25-dihydrolanosterol **23** is 32.5 μM) *(36)*. Aoyama has demonstrated that diol **8** is converted to diene **25** by a reconstituted system from *Saccharomyces cerevisiae (46)*, this conversion required two molar equivalents of NADPH. As with the rat liver system, diol **8** binds more tightly to the fungal enzyme than does 24,25-dihydrolanosterol **23** even though the V_{max} values for diol **8** and 24,25-dihydrolanosterol **23** are not significantly different.

In 1978, Akhtar *et al.* demonstrated the accumulation of 3β-hydroxylanost-8-en-32-al **10** under conditions of limiting NADPH concentrations *(48)*. In 1984, Trzaskos and coworkers verified these results and also identified diol **8** as a metabolite under similar conditions *(43,49)*. By utilizing rat liver microsomal preparations, Trzaskos *et al.* have established further conditions which promote the accumulation of demethylation intermediates **8** and **10** *(25,27,28)*. These include short incubation times, limiting enzyme amounts, high pH, high substrate concentrations, and inhibition with miconazole and ketoconazole. The major intermediate accumulated under all of the aforementioned conditions is the C-32 aldehyde **10** *(27)*. In addition, aldehyde **10** has been isolated from normal human lymphocytes cultured in the presence of human delipoproteinated serum *(50)*.

The lyase step of P-450$_{DM}$ involves the cleavage of the C-14 - C-32 bond and the loss of one of the protons at C-15. Early studies employing mevalonic acid stereospecifically labeled at C-2 demonstrated that the 15α-proton of lanosterol **18** is the one lost during the enzymatic formation of cholesterol *(51,52)*. In addition, the conversion of C-32 aldehyde **10** into diene **25** requires NADPH and O_2 *(48)*. With these facts in mind, numerous pathways for the last step of the 14α-demethylation of 24,25-dihydrolanosterol **23** have been put forth in the literature (Scheme 4). Pathway A involves 15α-hydroxylation followed by nucleophile-facilitated elimination *(53)*. This pathway has been discounted recently by Trzaskos *et al.* who have shown that synthetic 3β,15α-dihydroxylanost-8-en-32-al **29** is not metabolized to diene **25** under conditions which promote demethylation of lanost-8-ene-3,32-diol **8** and 3β-hydroxylanost-8-en-32-al **10** *(54)*. In addition, the 3β,15α-diol 32-aldehyde **29** was shown to be a time-dependent inhibitor of P-450$_{DM}$ which negates its consideration as an intermediate *(54)*. Pathways B and C proceed *via* a peroxyhemiacetal intermediate **30** which either decomposes directly to diene **25** (Pathway B) or rearranges in a Baeyer-Villiger fashion to generate a 14α-formyloxy species **31** which then undergoes a *syn* elimination (Pathway C) to provide diene **25** and formic acid *(44,55)*. Recent model studies reported by Vaz *et al. (56)* support the formation of an enzyme bound peroxyhemiacetal in P-450 mediated oxidative deformylations. These studies, along with those of Cole and Robinson *(57)* with aromatase, also lend support to the direct decomposition β-scission mechanism (*ie.* Pathway B). However, isolation of 14α-formyloxy-4,4-dimethylcholest-8-en-3β-ol **31** from the incubation of 3β-hydroxylanost-8-en-32-al **10** with rat liver microsomes suggests that Pathway C may constitute the natural mechanistic route *(54)*.

Dual-Action Inhibitors of Cholesterol Biosynthesis

Design of Dual Action Inhibitors of Cholesterol Biosynthesis. Oxysterols, such as those shown in Figure 1, reduce overall cholesterol biosynthesis and, hence, are of potential utility as antihypercholesterolemics. However, the inhibitory effects of these sterols are limited *in vivo* by their rapid metabolism. Pathways for the metabolism of the cholesterol analogs include oxidation at C-7 by 7α-hydroxylase and cleavage of the side-chain by enzymes involved in the biosynthesis of bile acids and pregnanes. 32-Oxylanosterols are intermediates in the removal of the 14α-methyl group by P-450_{DM} and thus are readily metabolized by this enzyme. The goal of the project described herein is to design, synthesize, and evaluate dual-action inhibitors of cholesterol biosynthesis that are more stable towards metabolism than the aforementioned oxysterols. These inhibitors were designed to cause both the inhibition of P-450_{DM} and the suppression of HMGR activity.

Compounds that are structurally similar to 32-oxylanosterols **8** and **10** are potentially useful inhibitors of cholesterol biosynthesis for the following reasons: 1) Being analogs of the natural intermediates generated by P-450_{DM}, they should act as competitive inhibitors of this enzyme. As noted earlier, both lanost-8-ene-3β,32-diol **8** and 3β-hydroxylanost-8-en-32-al **10** bind tightly to P-450_{DM} and thus inhibit the oxidative removal of C-32. 2) The competitive inhibition of P-450_{DM} by 32-oxygenated analogs of lanosterol may cause the accumulation of the natural intermediates **8** and **10**, as seen with miconazole, resulting in suppresion of HMGR activity. 3) Compounds similar in structure to lanost-8-ene-3β,32-diol **8** and 3β-hydroxylanost-8-en-32-al **10** may themselves cause suppression of HMGR activity. 4) Lanosterol analogs that cannot be deactivated by P-450_{DM} should exhibit reasonable metabolic stability since it is unlikely that lanosterol analogs will be substrates for 7α-hydroxylase or the side-chain cleavage enzymes.

With the above ideas in mind, the synthesis of compounds **32a**, **32b**, and **33** was undertaken. The 24,25-dihydro-Δ^7 compounds were chosen since they are synthetically more accessible than the 8,24-dienes. This should pose no problem since, as mentioned earlier, lanost-7-en-3β-ol **26** is a substrate for P-450_{DM} and the Δ^{24}-double bond is not necessary for the demethylation *(5-7,24)*. Also, as seen in Figure 1, both Δ^8- and Δ^7-lanosterol analogs have been shown to cause suppression of HMGR activity.

Compounds **32a**, **32b**, and **33** are methylated analogs of the intermediates of P-450_{DM} lanost-8-ene-3β,32-diol **8** and 3β-hydroxylanost-8-en-32-al **10**. Being similar in structure to the natural intermediates, compounds **32a**, **32b**, and **33** may bind tightly to P-450_{DM} and thus act as competitive inhibitors of this enzyme. In addition, they may mediate transcriptional and/or translational regulation of HMGR activity either directly or by causing the build-up of the natural intermediates **8** and/or **10**.

The C-14-C-32 bond of compounds **32a**, **32b**, and **33** should be stable to the lyase activity of P-450_{DM} by analogy to the similar compounds in the aromatase system (*ie.* compounds **34a**, **34b**, and **35**) *(58)*. Aromatase is the cytochrome P-450 monooxygenase that oxidatively removes the methyl at C-10 of androstenedione **36** to yield estrone **37** (Scheme 5) *(59)*. The methyl group is removed in a manner analogous to P-450_{DM}, *ie. via* hydroxylation, oxidation to the corresponding aldehyde, and removal of C-19 as formic acid. Compounds **34a**, **34b**, and **35** have been shown by Covey *(58)* to be competitive inhibitors of aromatase (K_i = 11 μM, 9.9 μM, and 150 nM for **34a**, **34b**, and **35**, respectively; K_m = 91 nM for androstenedione **36**). In addition, he has demonstrated that both of the diastereomers of **34** are oxidized to **35** by aromatase. However, the cleavage of the C-10-C-19 bond was not observed with any of these compounds *(58)*. Considering the similarity of aromatase and P-450_{DM}, it was anticipated that compounds **32a**, **32b**, and **33** will be stable to cleavage by P-450_{DM}.

Synthesis of Compounds 32a, 32b, and 33. Lanosterol analogs **32a**, **32b**, and **33** were prepared from lanosterol as shown in Scheme 6. Lanosterol **18** was

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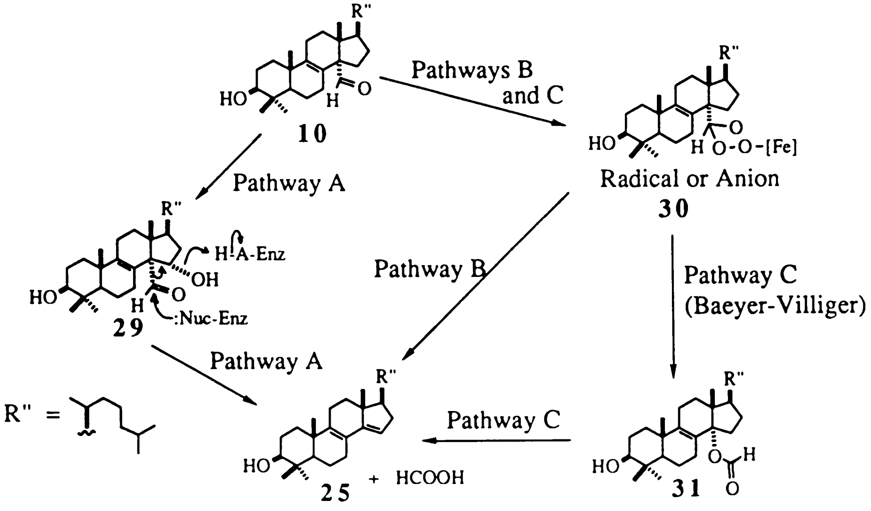
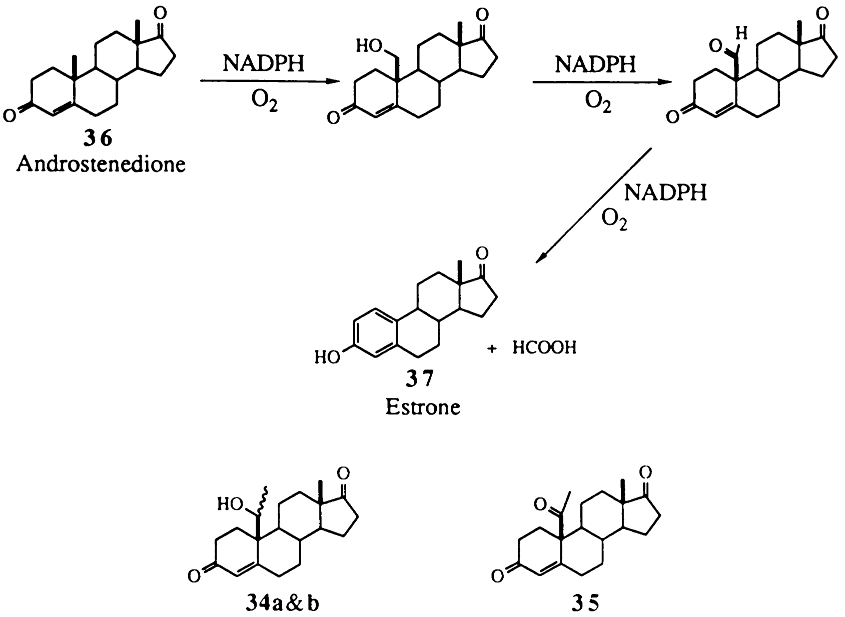

R"
HO
H
O
10
Pathways B and C
H O-O-[Fe]
Radical or Anion
30
Pathway A
H-A-Enz
OH
:Nuc-Enz
29
Pathway B
Pathway C (Baeyer-Villiger)
Pathway A
R" =
25
+ HCOOH
Pathway C
31

Scheme 4


36
Androstenedione
NADPH
O_2
HO
NADPH
O_2
O
H
NADPH
O_2
37
Estrone
+ HCOOH
34a&b
35

Scheme 5

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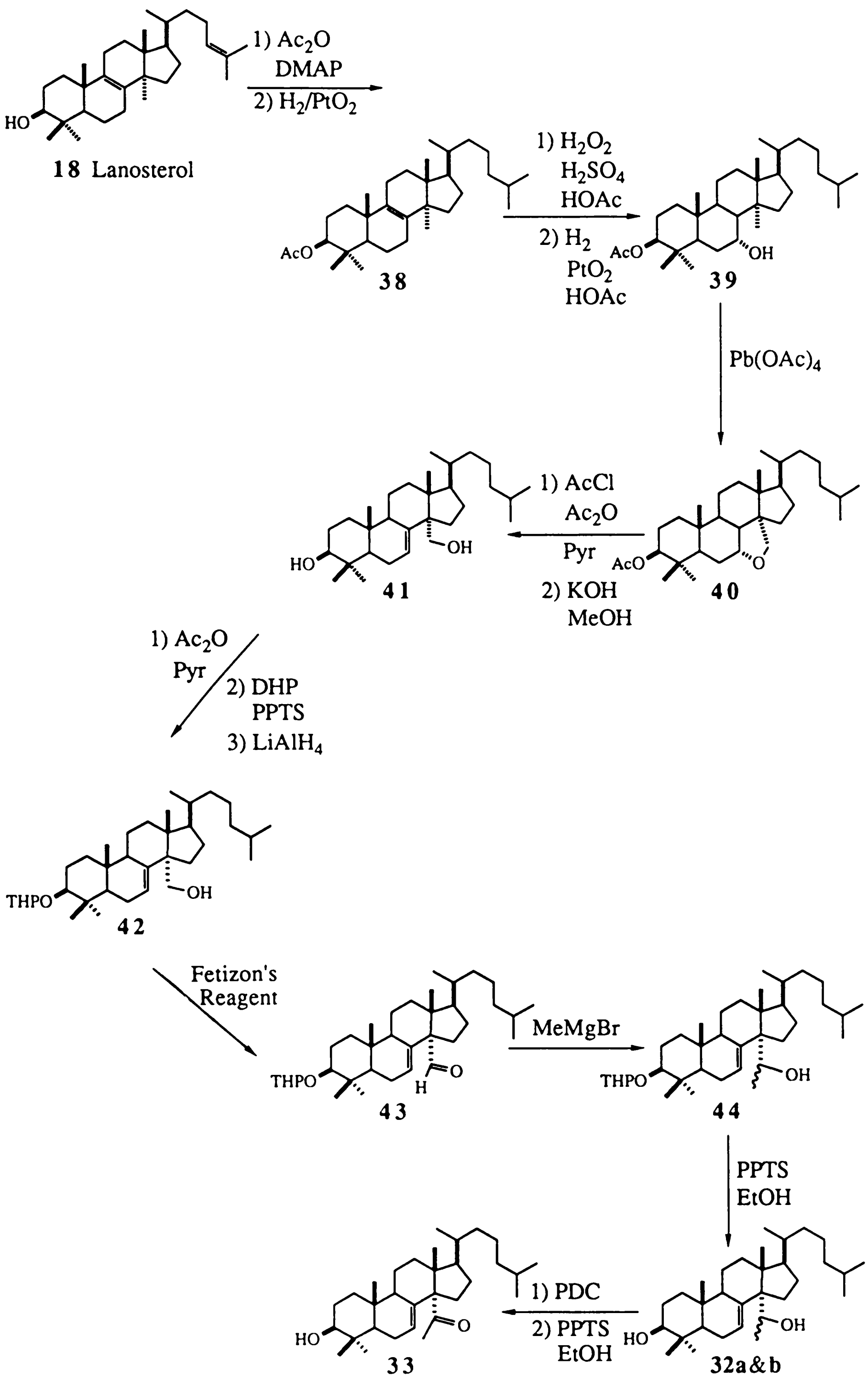
HO
18 Lanosterol
1) Ac_2O
DMAP
2) H_2/PtO_2
AcO
38
1) H_2O_2
H_2SO_4
HOAc
2) H_2
PtO_2
HOAc
AcO
OH
39
$Pb(OAc)_4$
AcO
O
40
1) AcCl
Ac_2O
Pyr
2) KOH
MeOH
HO
OH
41
1) Ac_2O
Pyr
2) DHP
PPTS
3) $LiAlH_4$
THPO
OH
42
Fetizon's
Reagent
THPO
H
O
43
MeMgBr
THPO
OH
44
PPTS
EtOH
HO
OH
32a&b
1) PDC
2) PPTS
EtOH
HO
O
33

Scheme 6

acetylated with acetic anhydride/DMAP followed by selective hydrogenation of the more accessible Δ^{24}-double bond to give 24,25-dihydrolanosteryl acetate **38**. Allylic oxidation of sterol **38** with H_2O_2/H_2SO_4/HOAc and hydrogenation under acidic conditions yields 7α-alcohol **39** as described by Pinhey *et al. (60)* and Sonoda *et al. (61)*. Lead tetraacetate mediated cyclization then results in formation of tetrahydrofuran **40** *(62)* which undergoes eliminative ring opening to give diol **41** after deprotection with KOH/MeOH *(45)*. Selective protection of the primary 32-alcohol as the acetate, protection of the 3β-alcohol as the tetrahydropyranyl (THP) ether, and removal of the 32-acetoxy moiety yields compound **42** which was oxidized with Fetizon's reagent to give aldehyde **43** *(45)*. The methyl group was then introduced by treatment with methylmagnesium bromide to yield compound **44**. This THP protected sterol could then either be deprotected to give desired methyl alcohols **32a** and **32b** as a mixture of diastereomers or oxidized with PDC and deprotectied to give methyl ketone **33**. The diastereomeric methyl alcohols **32a** and **32b** were easily separated by preparative HPLC.

$P\text{-}450_{DM}$ Inhibition Studies. The inhibitory properties of lanosterol analogs **32a**, **32b**, and **33** were assessed utilizing a modification of the assay developed by Trzaskos and coworkers *(43)*. Briefly, this assay quantitates the build-up of diene **25** by UV-HPLC. Since rat liver microsomal preparations are used, the pathways by which the diene is degraded must be inhibited. The reduction of the Δ^{14}-double bond of diene **25**, the next step in the cholesterol biosynthetic pathway, is inhibited by AY-9944 and the removal of the methyl groups at C-4 is blocked by the addition of cyanide. By carrying out incubations with 24,25-dihydrolanosterol **23** and varying concentrations of the methylated analogs **32a**, **32b**, and **33**, IC_{50} values for these compounds were determined. The results of these studies are summarized in Table I.

Table I: IC_{50} values for Compounds **32a**, **32b**, and **33**

Compound	IC_{50} Value
32a (Less polar diastereomer)	0.33 μM
32b (More polar diastereomer)	1.6 μM
33	9.1 μM

These preliminary results suggest that all three of the methylated lanosterol analogs are inhibitors of $P\text{-}450_{DM}$ (K_m of 24,25-dihydrolanosterol **23** is 33 μM under the assay conditions). Further evidence for the inhibition of $P\text{-}450_{DM}$ by sterols **32a**, **32b**, and **33** was provided by [^{14}C]acetate incorporation studies. In these experiments, the conversion of [^{14}C]acetate into cholesterol and lanosterol was determined in the presence of varying concentrations of inhibitor in exponentially growing cultures of CHO-K1 cells. The results of these studies are shown in Figure 2. All three of the methylated analogs cause a dramatic reduction in the incorporation of acetate into cholesterol with a concommitant increase in the production of lanosterol which is consistent with the inhibition of $P\text{-}450_{DM}$. As with the inhibition studies in rat liver microsomal preparations, the acetate incorporation studies in CHO cells indicate that the methyl alcohols **32a** and **32b** are more potent inhibitors of $P\text{-}450_{DM}$ than is the methyl ketone **33**. (The concentration of the methyl alcohols

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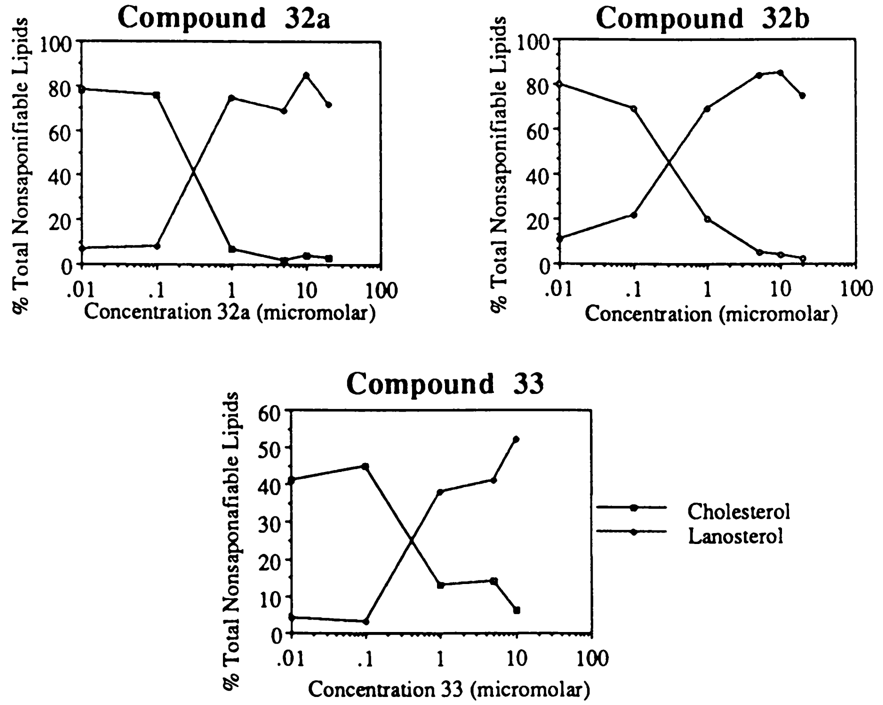

Figure 2. [^{14}C]Acetate incorporation studies.

required to reduce the amount of cholesterol to 50% of control is approx. 0.2 µM while that of the methyl ketone is approx. 0.5 µM.)

HMGR Activity Studies. An initial screening of the effects of compounds **32a**, **32b**, and **33** on HMGR activity in CHO cells indicate that all three compounds cause a reduction in activity compared to control cells (Figure 3). HMGR activity was determined in permeabilized cells by monitoring the conversion of [^{14}C]HMG-CoA to [^{14}C]mevalonate, using [^{3}H]mevalonolactone as an internal standard *(63)*. Addition of compounds **32a**, **32b**, and **33** to CHO cell sonicates had no effect on HMGR activity, demonstrating that these compounds are not direct inhibitors of HMGR. The results of concentration studies of compounds **32a**, **32b**, and **33** versus HMGR activity in both normal CHO and P-450$_{DM}$-deficient (AR45) cells are shown in Figure 4. All three compounds mediate suppression of HMGR activity in CHO cells. The concentrations of inhibitor required to reduce HMGR activity to 50% of control are approx. 15 µM, 2 µM, and 1 µM for lanosterol analogs **32a**, **32b**, and **33**, respectively. The marked difference between the suppression of HMGR activity in AR45 cells versus CHO cells for all three compounds suggests that the main mechanism of suppression may be the competitive inhibition of P-450$_{DM}$ causing the build-up of the natural intermediates **8** and **10** which in turn suppress HMGR activity.

Conclusions and Comments

Methylated lanosterol analogs **32a**, **32b**, and **33** have been found to inhibit P-450$_{DM}$ and to cause the suppression of HMGR activity and thus, they are dual-action inhibitors of cholesterol biosynthesis. Further experiments to ascertain the absolute

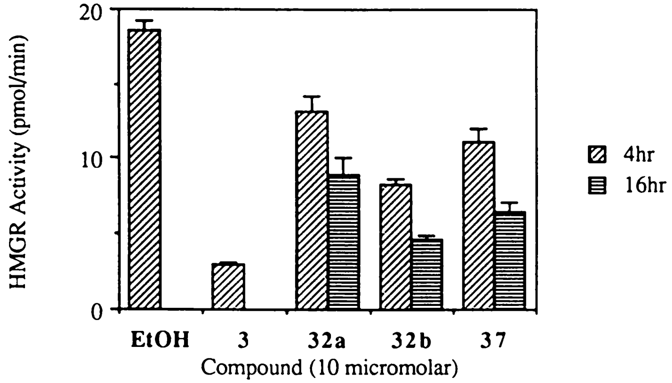

Figure 3. Inhibition of HMGR activity by Compounds **32a**, **32b**, and **33**.

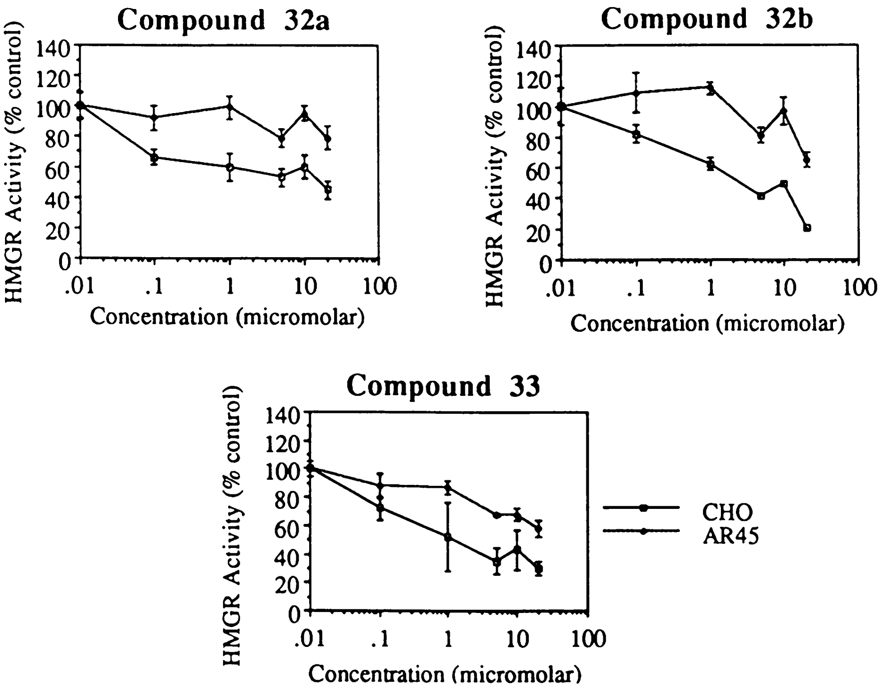

Figure 4. HMGR suppression studies.

configuration of diastereomers **32a** and **32b** are underway along with studies to determine whether or not compounds **32a**, **32b**, and **33** are metabolized by P-450_{DM}.

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Chapter 10

Regulation of Sterol Biosynthesis and Its Phylogenetic Implications

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only the fit survive!
Charles Darwin, 1859

Sterols are ubiquitous natural products found throughout the evolutionary hierarchy from cyanophytic bacteria to tracheophytes and man. In this article regulation of sterol biosynthesis is examined with special emphasis on kinetic and thermodynamic control of the pathway and its importance to evolution.

In contrast to animals and fungi where cells usually synthesize and accumulate a single sterol end product-cholesterol and ergosterol, respectively, the plant cell synthesizes a cocktail of sterols which differ structurally from one another primarily in the extent to which the side chain is dehydrogenated at positions C-22 and C-25 and alkylated at position C-24 (*1-3*). This finding presents a challenge to chemotaxonomists and that is the need to explain sterol diversity in terms of evolution. We have stated elsewhere (*4, 5*), it seems unlikely that random mutations followed by natural selections (of enzymes that act on the sterol substrate) are the driving force for phenotypic change in the sterol structure. We have argued in favor of a teleonomic principle where mutations in the sterol pathway are regulated by the function(s) played by the end product sterol, e.g., by membrane feed-back signals (*4-6*). These signals are responsible to channel, by thermodynamic control of the pathway, then limit, by the fit of the sterol in the membrane, the outcome of biosynthesis. It follows that where the structure and amount of 24-alkyl sterols vary in plants, the functional integrity of the membrane may also vary; as such the regulatory mechanisms which control carbon flow through the pathway become phylogenetic determinants for the production of what might be viewed as "good" and "bad" membrane inserts. These architectural components may affect evolution by influencing cellular vitality and reproductive fitness. As a continuation to our earlier thoughts on the evolution of sterols, we discuss herein a new concept that there may exist phylogenetic significance for the

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operation of taxa-specific, kinetically favored pathways in sterol genesis and a model has been proposed for a single enzyme complex which may be responsible for the diversity of 24-alkyl and 24-desalkyl sterols isolated from nature. For the interested reader, there are several excellent reviews on regulation of sterol biosynthesis, but they markedly differ from the points of view expressed here (*7-11*).

Critical to our discussion of sterol biochemistry is an operating definition for a sterol. Remarkable as it may seem, there exists much confusion in the literature regarding the features that define sterol structure and those features which distinguish a sterol from sterol-like molecules that possess the same or similar constitution. Thus, we begin with an examination of the various definitions for sterol and then examine the rules by which structure and stereochemistry may be defined.

A "sterol" may be defined by using a combination of structural and biosynthetic reasoning (*1*). As often thought, a sterol is any unsaponifiable steroid alcohol with an aliphatic side chain of 8 to 10 carbon atoms and a hydroxyl group at C-3 with lipid properties (*1, 12*). This definition suits their chemical classification based on the cholestane skeleton and the distribution data of sterols prior to about 1977 (*13*). The definition, however, fails to allow for the existence and naming of highly bioalkylated side chain sterols recently discovered in plants (*13*), fungi (*14*) and marine organisms (*15*), and fails to distinguish sterols from sterol-like molecules which have the same molecular constitution, e.g., tetracyclic triterpenoids, such as, dammardienol. Natural product chemists recognize all 30-carbon polycycles with geminal methyls at C-4 in ring-A as triterpenoids, including lanosterol and cycloartenol. The C-4 carbon atoms are numbered C-28 and C-29 for tetracycles (*16-18*) or C-23 and C-24 for pentacycles (*19, 20*). Steroid biochemists view lanosterol and cycloartenol as sterols and number the geminal methyls at C-4 as C-30 and C-31 (*1, 12*).

Another, albeit circular, definition for a sterol is given by chemists (*18*). The definition is based on the term for being a steroid (oid=like): it must possess the skeleton of cyclopenta[a]phenanthrene or a skeleton derived therefrom by one or more scissons or ring expansions or contractions. Methyl groups are normally present at C-10 and C-13. An alkyl side chain may also be present at C-17. Sterols are steroids carrying a hydroxyl group at C-3 and most of the skeleton of cholestane. Additional carbon atoms may be present in the side chain. Whereas this definition fails to fully distinguish a sterol from sterol-like triterpenoids, the system nevertheless allows for some tetracyclic triterpenoids, e.g., lanosterol to be numbered with the steroid numbering system.

An operational definition for the term "sterols" to which we ascribe is: any chiral tetracyclic isopentenoid which may be formed by the cyclization of squalene oxide through the transition state possessing stereochemistry similar to the trans-syn-trans-anti-trans-anti configuration i.e., the protosteroid cation, and retains a polar group at C-3 (hydroxyl or keto), an all-trans anti stereochemistry in the ring system and a side chain 20R-configuration (*1*). Molecules that fail to incorporate these stereochemical features may be considered sterol-like molecules. It follows further

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for functional reasons that sterol-like molecules should not replace sterols in the biosynthetic pathway and undergo transformations by enzymes that evolved to act on the sterol substrate, although they may mimic other functions of sterol, e.g., as membrane inserts, because they possess similar amphiphilic properties. Thus, 20-epilanosterol and euphol, which are isomeric with lanosterol should be considered on biosynthetic grounds as sterol-like molecules. On architectural grounds, these and related isomers and pentacyclic analogs of sterols, e.g., isoarborinol relative to parkeol (*21*), may also be considered as sterol-like molecules (*4, 22*).

Structure and Stereochemistry

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One set of rules for nomenclature of steroids has been defined by the joint IUPAC commission on the nomenclature of organic chemistry and IUB commission on biochemical nomenclature (hereafter JCBN rules) in a revised report published in 1989 where the numbering and stereochemistry is based strictly on the geometry of the molecule (*18*). The JCBN rules discuss the stereochemical description (R/S system) and numbering of the carbon atoms in the ring and side chain based on the cholestane skeleton, as shown in Figures 1 and 2. The revised system renumbers the nuclear methyl groups and incorporates a higher-order labelling for added methyls attached to C-24 rather than numbering them outright as they have been in the past. One problem with the revised report is that it fails to allow for the numbering in sequence of geminal methyls attached to C-24 i.e., as in 24-dimethyl cholesterol. In other words which of the two C-atoms is to receive the lower ordered prime number, the one in front or in back of the plane? Another set of rules pertaining to the systematic naming and stereochemical nomenclature for specifically, the side chain was developed by phytochemists studying plant sterol metabolism (*23*). We now refer to this as the "biosynthetic side chain rule" which has obtained various shades of meaning (cf. *23-25*) and which may employ either the R/S- or α/β-descriptors for chiral groups in the side chain. A recognition of which rule is used is not trivial particularly with respect to NMR and biosynthetic studies where comparisons of chemical shift data are made in tables that describe specific positions for each carbon atom and in studies where isotopically labelled precursor molecules, such as, mevalonic acid are fed to cells for a determination of their carbon atoms' biosynthetic destination in the sterol molecule (*25, 26*).

The difference regarding side chain nomenclature in the two numbering systems is primarily with the numbering of the terminal carbon atoms and of the stereochemistry of substituents in the side chain. Both rules use the convention where the backbone of the side chain at C-17 is oriented into the right-handed and staggered conformation. In the JCBN rules C-26 and C-27 are viewed as chemically equivalent even though they are magnetically (in NMR) and biochemically distinct carbon atoms. Regardless of whichever of the two terminal methyl groups attached to C-25 is substituted, e.g., hydroxylated, that methyl is assigned by the lower number (C-26) and the other C-atom becomes C-27-no stereochemical or biochemical distinction between the two groups is to be implied. When one of the two terminal isopropyl groups becomes unsubstituted or unsaturated, the two methyl groups attached at C-25 are then numbered with, the

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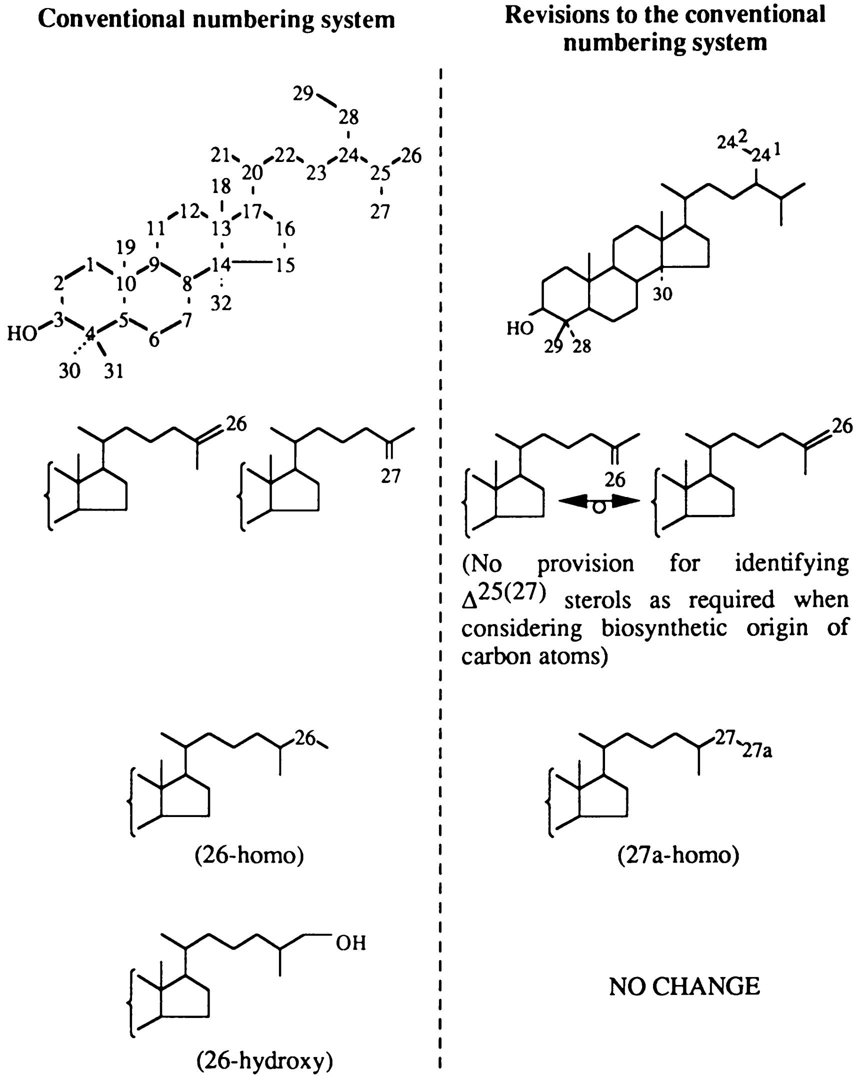
Conventional numbering system
Revisions to the conventional numbering system
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16
19
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9
14
15
2
10
8
32
HO
3
4
5
6
7
30
31
26
27
(26-homo)
(26-hydroxy)
OH
24²
24¹
30
HO
29
28
26
26
(No provision for identifying $\Delta^{25(27)}$ sterols as required when considering biosynthetic origin of carbon atoms)
27
27a
(27a-homo)
NO CHANGE

Fig. 1. Comparison of the conventional and revised conventions for numbering of the carbon atoms of the sterol skeleton based on the JCBN rules.

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carbon bearing the double bond as C-26 as in 25(26)-double bond (cf. Fig. 1). The revised JCBN rules suggest that when an unsubstituted 25(26)-bond is present the two methyl groups attached at position 25 are numbered 26 and 27 with the lower number (*26*) for the methyl group trans to position C-23. However, the illustration given in the report indicates the set of unsubstituted carbon atoms are cis to C-23. Therefore, to be consistent, the proper view of the molecule should be as shown in Figure 1 where rotation about C-25 has occurred.

Also evident from Figure 1 is that the revised JCBN report suggests that when C-26(27) is modified by the addition of a hydroxyl group compared with a methyl group the numbering system changes: homologzation of the side chain is treated so that the extra methyl group is attached to C-27 rather than C-26. In the biosynthetic side chain rule C-26 is defined as the side chain carbon atom which is derived from C-2 of mevalonic acid whereas C-27 is derived from C-6(C-3') of mevalonic acid. When the side chain possesses the 24,25-double bond, e.g., as in lanosterol and cycloartenol, the (Z)-methyl group is derived from the cis methyl in squalene and C-6 of mevalonic acid whereas the (E)-methyl group is derived from the trans methyl in squalene and C-2 of mevalonic acid. By these rules C-2 of mevalonic acid may never become C-27 (Fig. 3). By a combination of ^{1}H- and ^{13}C-NMR it is possible to precisely define the chemical shift of C-26 and C-27. Thus, from 2D NOE experiments on 10α-cucurbitadienol (which is derived via the protosteroid cation and is an isomer of lanosterol with a Δ^{24} bond) we have observed that H-24 was correlated to the downfield vinyl methyl at δ 1.68 making that methyl cis to H-24 but trans to C-23. The carbon on the (E)-methyl group incorporates label from C-2 MVA (*27*), therefore, it becomes C-26. The downfield signal at δ 25.7 in the carbon spectra is correlated in the proton spectra to the down field singlet at δ 1.687. Further confirmation by an NOE difference experiment was by irradiation of δ 1.68 and 1.60. An enhanced peak was observed at δ 5.09 only by irradiation of the downfield methyl (*27*). However, with no regard to biosynthetic origin, JCBN convention dictates that when these terminal groups are distinguished by isotopic labelling the labelled methyl group assumes priority over the unlabelled group methyl group. Thus, whichever the carbon labelled from either C-2 or C-6 mevalonic acid (separate preparations) will be designated C-26. For the nonchemist who wishes to study biosynthesis the JCBN rules present difficulties in tabulations of carbon signals in NMR after incubations with ^{13}C-mevalonates and presenting logical discussions on biochemical mechanisms related to the side chain (cf. *25*).

What is or should be the preferred stereochemical nomenclature for the side chain is another area of much confusion and lack of agreement. The recommendation by the JCBN is to use the sequence rules which employ the R/S- and E/Z-nomenclature (*18*). However, these rules have not always been enforced as evidenced by papers that appear in chemical journals with an alternative system that describes the stereochemistry of the side chain viz., the α/ß- and cis/trans-nomenclature. The main advantage to the use of the latter system is that the R/S nomenclature employs excessive empirical "priorities" so that two stereochemically similar compounds which have different substituent patterns associated with the same site may have reverse configurations - from R to S (with no inversion to be

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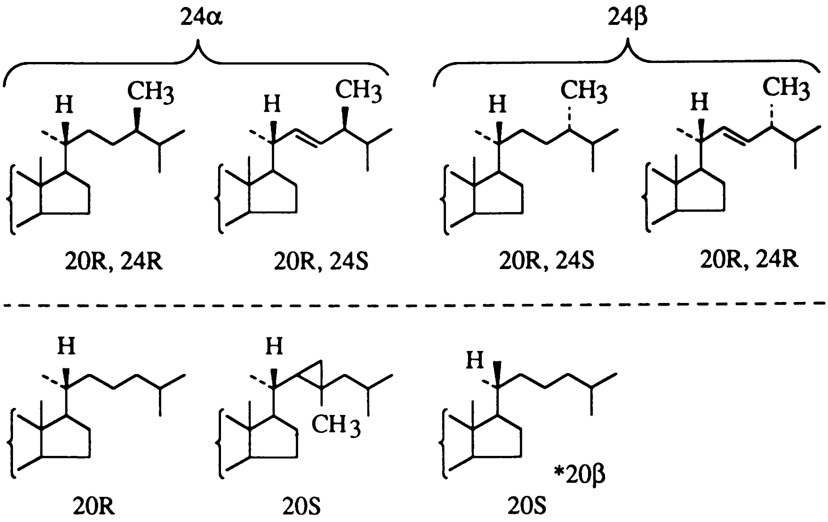
24α
24β
H
CH3
20R, 24R
20R, 24S
20R, 24S
20R, 24R
H
H
CH3
H
20R
20S
*20β
20S

Fig. 2. Comparison of the R/S and α/ß notations for describing side chain chiral centers.

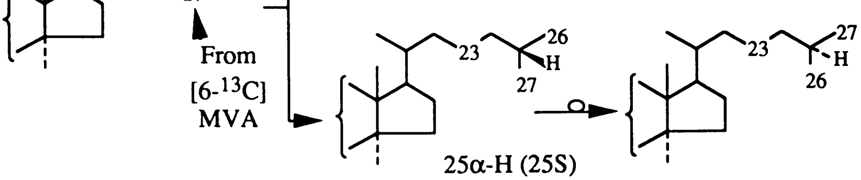
From
[2-¹³C]
MVA
H
23
26
27
From
[6-¹³C]
MVA
23
26
H
27
25β-H (25R)
23
27
H
26
23
26
H
27
25α-H (25S)
23
27
H
26

Fig. 3. Carbon numbering based on the biosynthetic destination of C-2 and C-6 mevalonic acid into the sterol side chain.

implied), as at C-20, e.g., whether there are or are not multiple groups at C-22, at C-24, e.g., whether there is or is not a Δ^{22} bond, and at C-25, e.g., when C-26 is either hydroxylated or contains a isotopically labelled ^{13}C carbon atom (Figures 4 to 6). Alternatively, the α and ß notations are independent of substituents at neighboring atoms - α in front of the plane and ß in back of the plane.

The face of the double bond is designated si or re and is dependent on the substituent pattern associated with the trigonal center. As shown in Figure 6 the re-face may become the si-face depending on the presence of adjacent double bonds in the side chain. Thus, two geometrically similar compounds may present the same face of the double bond to a catalyst but with opposite nomenclature. The same reversals imposed by the sequence rules operate for the pro-R/S notation - note, for example, the effect of adding a methyl group to C-24 on the prochiral description for the C-23 hydrogen atoms (Fig. 5). The term prochiral is used for a compound that has two enantiotopic atoms or groups. A formally symmetric atom or group in the sterol side chain after its modification e.g., replacement of carbon-12 with carbon-13 at C-26, may lead to an R-chiral center. The carbon atom that is to produce this chirality at C-25 is called the pro-R carbon. The other is pro-S (Figures 3 and 4). Reversals in stereochemical assignment for the same geometry also operate in the use of E/Z-nomenclature cf. Figure 5, which can be avoided by the cis/trans system.

The numbering system by JCBN also fails to fully realize a system which can number the complicated multiple bioaklylated side chains. Djerassi and coworkers have suggested an alternative approach and that is to number the alkyl groups by the biosynthetic order in which they were introduced (*28*). This system is not fully satisfactory at the present time but with additional knowledge of alkylation biosynthesis this procedure may prove to have merit (Fig. 7).

Conformational Analysis

There are two different approaches to view the sterol molecule i.e., the conventional and conformational view. When the sterol molecule is viewed on edge to assess its 3-dimensional geometry and conformation, as shown in Figure 8, a different perspective view of the molecule is introduced from that which is illustrated in its conventional representation shown in Figure 1. By defining the planes that project from the internuclear line that connects the nucleus and side chain, the global shape of the sterol, including top and bottom faces of the molecule may be defined, as discussed in what follows.

With the nucleus as a reference point it becomes possible to establish connecting lines and planes of stereochemical demarcation and confluence between the nucleus and side chain and along the side chain. As shown in Figure 8, the sterol molecule assumes a defined volume which is determined by its length (oriented from proximal to distal end or from C-3 to C-26(27)), height (top to bottom or from C-18 to C-32) and width (side-ventral, to side-dorsal, or from C-10 to C-5 or C-5 to C-10, respectively). The magnitude of the volume is a function of the population of side chain rotamers which assume the right-handed staggered conformation, the presence of angular methyl groups which protrude from the ß-

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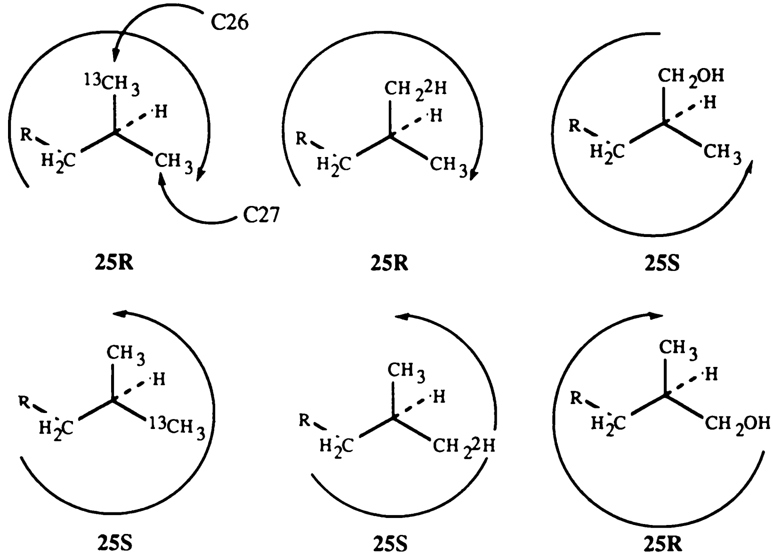
C26
13CH3
CH2 2H
CH2OH
H
R
H2C
CH3
C27
25R
25R
25S
CH3
13CH3
CH2 2H
CH2OH
25S
25S
25R

Fig. 4. Application of the JCBN rules to the numbering of carbon atoms and defining chirality at C-25 of the sterol side chain.

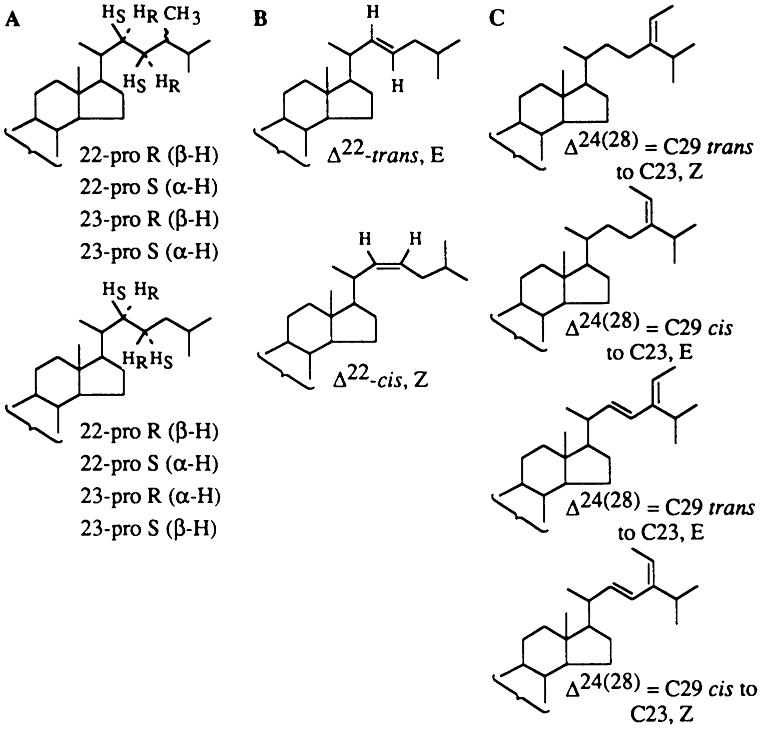
A
HS HR CH3
HS HR
22-pro R (β-H)
22-pro S (α-H)
23-pro R (β-H)
23-pro S (α-H)
HS HR
HR HS
22-pro R (β-H)
22-pro S (α-H)
23-pro R (α-H)
23-pro S (β-H)
B
H
H
Δ22-trans, E
H H
Δ22-cis, Z
C
Δ24(28) = C29 trans to C23, Z
Δ24(28) = C29 cis to C23, E
Δ24(28) = C29 trans to C23, E
Δ24(28) = C29 cis to C23, Z

Fig. 5. Comparison of the JCBN rules with the α/β and cis/trans systems to describe stereochemistry in the sterol side chain.

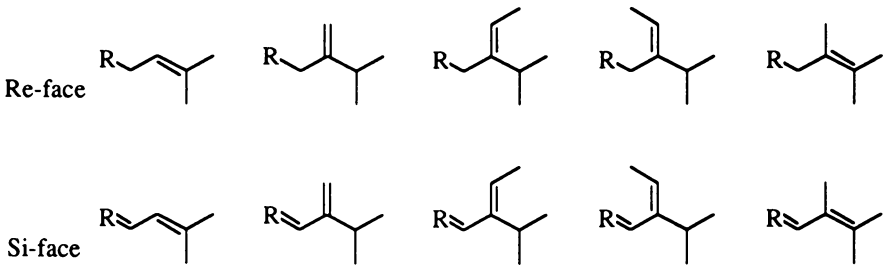
Re-face
R
R
R
R
R
Si-face
R
R
R
R
R

Fig. 6. Comparison of the re- and si-faces of the trigonal center at C-24 of the sterol side chain containing one or two double bonds.

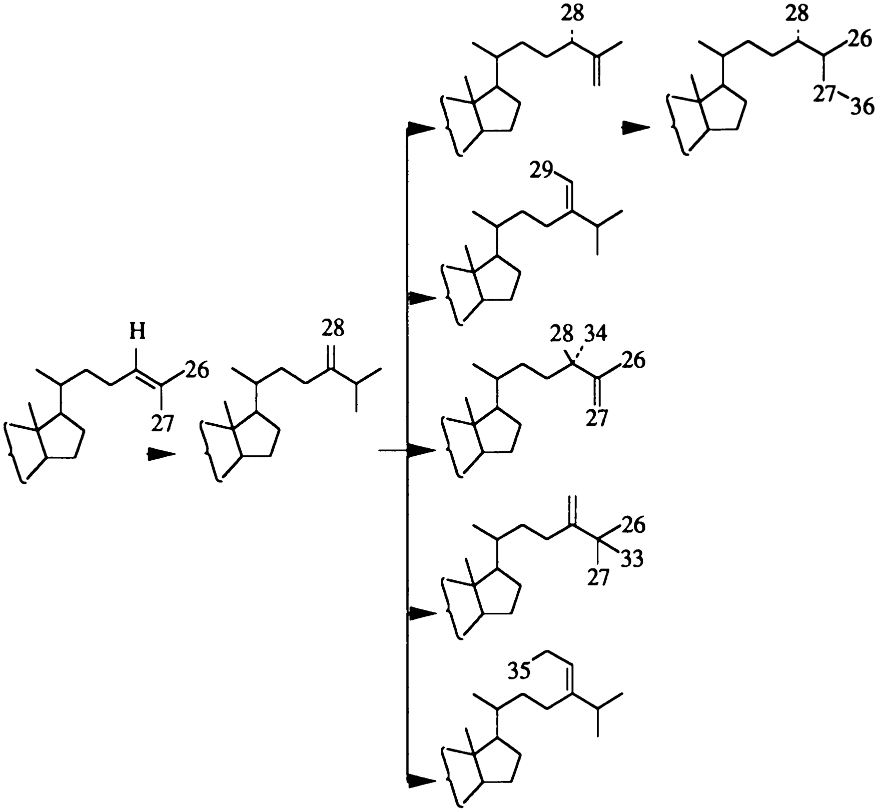
28
28
26
27
36
29
H
26
27
28
28
34
26
27
26
33
27
35

Fig. 7. Various approaches to the numbering of a highly bioalkylated sterol side chain based on the biosynthetic order in which they were introduced. See text for a key to the literature.

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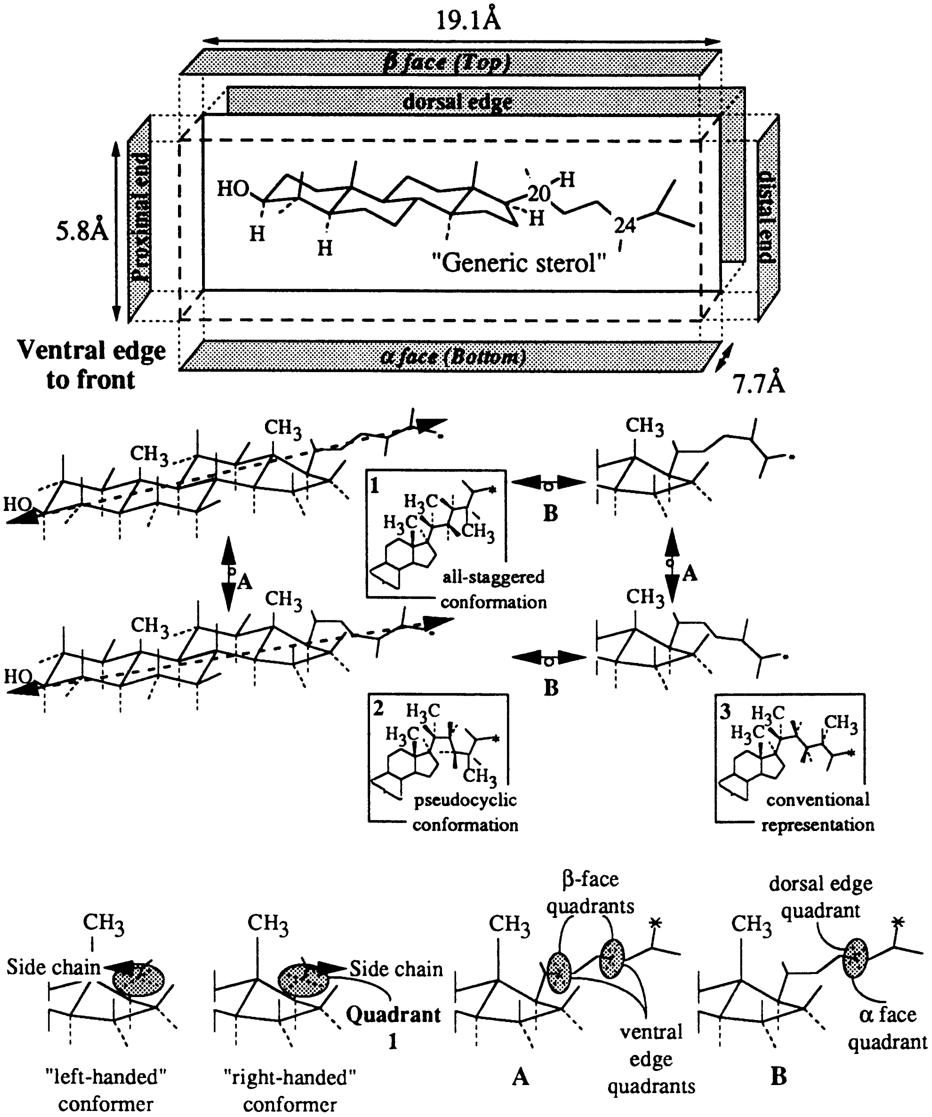
19.1Å
β face (Top)
dorsal edge
Proximal end
distal end
5.8Å
HO
H
H
20
H
H
24
"Generic sterol"
Ventral edge to front
α face (Bottom)
7.7Å
CH3
CH3
HO
A
B
1
H3C
H3C
CH3
all-staggered conformation
2
pseudocyclic conformation
3
CH3
conventional representation
CH3
Side chain
"left-handed" conformer
Side chain
Quadrant 1
"right-handed" conformer
β-face quadrants
ventral edge quadrants
A
dorsal edge quadrant
α face quadrant
B

Fig. 8. Conformational perspective of a generic sterol with internuclear line shown. Inserts represent equivalent conventional views of a C-24 methylated side chain.

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and α-faces and the degree to which the side chain sweeps out a cone. The molecular dimensions for the generic sterol given in Figure 8 are derived from the literature (*22*). The dimensions are based on the sterol assuming the preferred conformation with the side chain to the right in the all staggered conformation; it does not give the maximal and minimal volumes attainable for a sterol.

The side chain may assume different rotamers which may place the aliphatic chain into different quadrants of space. For 24-alkyl sterols with the 20-R configuration, the median side chain conformation is the right-handed (C-22 trans to C-13) structure that is staggered and coplanar with the nucleus. Of the four equatorial spatial quadrants the side chain may orient (*29*), the side chain of 20-R sterols orients principally into Quadrant 1, in both solution and the solid state (*27, 30-33*). The preferred right-handed conformation is the result of 1,3-diaxial interactions between H-20 and C-13 (*29, 30-32*). The chiral C-24 methyl group which flexes primarily in Quadrant 1 (Fig. 8), may orient into an equatorial and ventral (with respect to the nucleus) orientation, as shown in Figure 8, or project toward either the α- or ß-face (with respect to the nucleus). The side chain may also assume either the all-staggered or pseudocyclic conformations which continue to be coplanar with the nucleus but do not affect the spatial disposition of angular groups attached to C-24. For instance, the solid state conformation of ergosterol (*34*), indicates the direction of the 24ß methyl group is ventral and the H-atom projects to the α-face (with respect to the nucleus). The illustration of solid and dotted lines for the side chain groups in the conformational perspective should not be confused with similar notations designating absolute stereochemistry in the conventional view.

Uniformity Versus Variability in Sterol Structure

In certain ways all sterols are the same, and in others they are very different. Based on the natural distribution of sterols most of their mass is associated with membranes (*1*). The molecular features of sterols to fit membranes are that they possess an unsubstituted C-3 hydroxyl group, a planar tetracyclic nucleus and an intact side chain of 7 to 11 carbon atoms (*1, 4, 35, 36*). The variability in sterol structure is primarily associated with the different side chain compositions which are obtained by bioalkylation. The uniformity in sterol content is with the nature of the squalene oxide cyclization products. Of the tetracyclic and pentacyclic products which are formed by the cyclization of squalene and its epoxide only a select few products enter into the sterol pathway and fewer less actually become transformed into suitable membrane inserts during active cell proliferation. The two principal cyclized products of squalene oxide that appear to first enter into the sterol pathway are cycloartenol and lanosterol.

The Cycloartenol-Lanosterol Bifurcation

Investigations which have been concerned with the phylogenetic distribution of sterols in nature have drawn attention to a two pathway model based on the outcome of the cyclization of squalene oxide and the chirality and extent of alkylation of the

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C-24 alkyl group in the side chain. Organisms which have evolved from a photosynthetic lineage are thought to operate a cycloartenol based pathway whereas organisms which have evolved from a non-photosynthetic lineage operate a lanosterol-based pathway (*2, 36-40*). Additionally, organisms which introduce a ß-methyl group are more primitive than organisms which introduce an α-ethyl group at C-24 (*1*). Thus, the model shows that lanosterol gives rise to ergosterol in fungi and cholesterol in animals whereas cycloartenol gives rise to stigmasterol in plants and poriferasterol in algae, as shown in Figure 9. Marine organisms and insects are thought to lack de novo sterol synthetic capability and acquired their nutritional sterol from the diet (environment) (*1*). Prokaryotes, are thought not to synthesize sterols but for a different reason than insects and marine organisms. The latter two are thought to have possessed the sterol pathway then lost part of it during evolution. The former never possessed it because the earth was presumably anaerobic during the early phases of prokaryote evolution, so that the aerobic steps involved in, for instance, squalene epoxidation and sterol demethylation could not occur in the primal organisms. It is assumed that the extant prokaryotes lack the information to carry out oxygenic metabolisms and therefore cannot produce sterols (*22, 35*). In order for them to survive and evolve they operate another pathway where sterol-like pentacyclic triterpenoids are formed by the anaerobic cyclization of squalene (*1, 22*). The evolution of the sterol pathway has been discussed elsewhere (*2, 4, 22*). A typical phylogenetic derivation whereby sterol production follows hopanoid (pentacycles) production in evolution is shown in Figure 9.

The C-24 Alkylation-Reduction Bifurcation

The variant outcomes of the metabolism of the Δ^{24} bond gives rise to the diversity of Δ^5 sterols in plants, where the production of the Δ^5 grouping represents functional completion of the pathway (i.e., for nuclear transformations). Akihisa et al., have listed 60 different phytosterols with modified side chains (*37*). With the advances in chromatography and nuclear magnetic resonance spectroscopic techniques, fungi have recently been found to produce a variety of C-24 alkylated sterols (*14, 38, 39*). The variation in sterol composition is now known to be ontogenetically regulated in plants (*40, 41*), so that C-24 alkyl epimers may appear in the same plant, but at different developmental stages (*42*). However, C-24 α-ethyl phytosterols which were thought to occur only in tracheophytes have recently been detected in algae, e.g., sitosterol and stigmasterol (*43*), and conversely, C-24 ß-ethyl sterols which were thought to be unique to algae, e.g., clionasterol and poriferasterol, have been shown to occur in vascular plants (*40*). Thus, the variations in sterol structure in nature appear to be associated with the formation of end products that arise by the C-24 alkylation pathway.

The blueprint for the mechanism of C-24 alkylation was discovered by Castle et al. (*44*). About ten years later, Malhotra and Nes drew attention to the mechanistic similarities in the saturation of the 24,25-double bond by CH_3^+ and H^+ (*45*), as shown in Figure 10. Nes and coworkers then established the functional necessity for introduction and stereochemistry of the C-24 alkyl group in yeast

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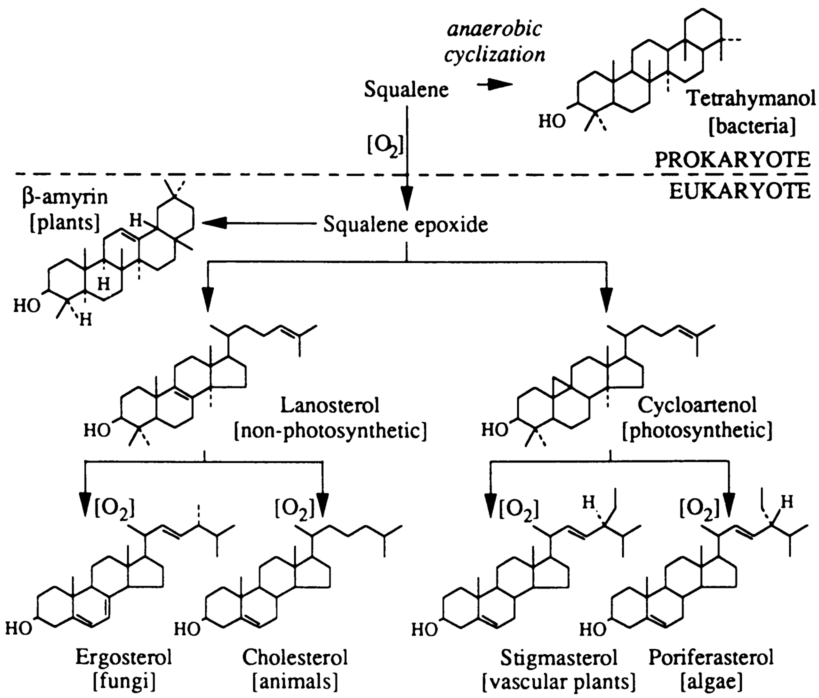
anaerobic cyclization
Squalene
Tetrahymanol
[bacteria]
[O2]
PROKARYOTE
EUKARYOTE
β-amyrin
[plants]
H
H
HO
H
Squalene epoxide
Lanosterol
[non-photosynthetic]
HO
Cycloartenol
[photosynthetic]
HO
[O2]
[O2]
[O2]
H
[O2]
H
HO
HO
HO
HO
Ergosterol
[fungi]
Cholesterol
[animals]
Stigmasterol
[vascular plants]
Poriferasterol
[algae]

Fig. 9. Cycloartenol-lanosterol bifurcation in sterol biosynthesis.

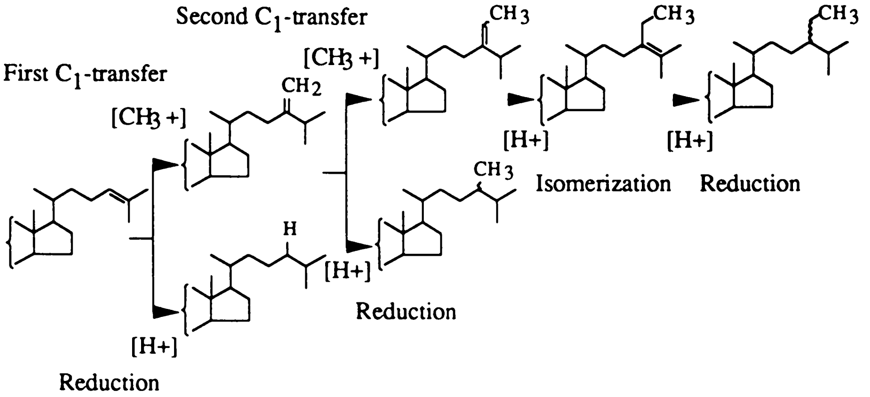
Second C1-transfer
First C1-transfer
[CH3 +]
[CH3 +]
CH2
CH3
CH3
CH3
[H+]
[H+]
CH3
Isomerization
Reduction
H
[H+]
Reduction
[H+]
Reduction

Fig. 10. C-24 Alkylation-reduction bifurcation in sterol biosynthesis.

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which naturally produces 24-methyl sterols by incubating yeast under anaerobic conditions in the presence of various sterol supplements (Table 1) (*46*). There is confirmatory evidence for the physiological importance for the biosynthetic inclusion of the C-24 methyl group into the sterol side chain from studies with other fungi (*47, 48*) and plants (*49, 50*).

Goad and coworkers (*51*) and others (*25, 26, 52-54*) have pursued a more detailed mechanistic inquiry into the face of methyl attack on the double bond (Fig. 11). The methylation reaction determines the chirality at C-24 for some but not all sterols. For some sterols, such as sitosterol the saturation of a 24,25-double bond determines the chirality at C-24. Of added interest is the resulting outcome at C-25 after saturation of the 24,25-double bond. In plants and fungi the configuration at C-25 is 25-S (*26, 52*), whereas in animals and perhaps insects it is 25-R (*25*). It is unclear whether an inversion mechanism operates in some organisms. Nevertheless, animal cholesterol and plant cholesterol may become stereochemically different molecules.

Common to the formation of all three products of methylation is the intermediacy of the transition state structure (Fig. 12) from which the initial C-25 carbenium ion high energy intermediate (HEI) is formed. Chemically, the initial formation of the C-25 carbenium ion is favored by hyperconjugative interaction of the charge at C-25 with the six H-atoms at C-26 and C-27, so that a 25(27)-sterol might be the expected product of the reaction (assuming the enthalpic barrier to direct loss of a proton is less than that associated with a 1,2-hydride migration), followed by lesser amounts of other stabilization products of the C-25 carbenium ion.

When not at equilibrium and cofactors are not limiting, the outcome of methylation is governed by kinetic control. Entropic considerations provide leading information on the structure reactivity and specificity relationships involving discrete changes in the structure of the substrate. Pursuant to an understanding of the free-energy change that proceeds with catalysis is a knowledge of the conformational dispositions of the ground state structures of substrate and product(s) and transition and HEI structures that are formed along the reaction pathway. Also relevant is a recognition that the transition state and HEI structures are regarded as a dipolar species for which the developing charge at C-25 is stabilized by electrostatic interaction with one or more counterions strategically located in the catalytic site.

We have considered the relative free energies of the HEIs and transition states of cyclopropyl sterols generated during the course of C-24 methylation in plants, as illustrated in Figure 12. The free energy diagram is based on the observed ratio of cyclosteroid C-24 methylation products that are experimentally obtained in plants - 24(28)methylene cycloartanol (P_a) > cyclolaudenol (P_b) > cyclosadol (P_c) (*55-57*) and the K_i values for inhibitors assayed in a cell-free sunflower system which we designed as isoelectric HEI analogues of the two carbocations formed during methylation (Fig. 12) (*58*).

The importance of the global recognition by the enzyme for the acceptor molecule and how it manifests the rate of reaction is central to enzyme kinetics. For mechanistic enzymology involving sterols, the role of the sterol conformation in catalysis has generally not been considered, except for the difference in activity

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Table 1. Effect of Sterol Structure on the Growth of New Cells

Sterol Supplement	*Percent Growth*[a]
5α-Cholestane	< 1
5α-Cholestan-3ß-ol	23
Cholesterol	23
Lathosterol	38
7-Dehydrocholesterol	23
24α-Methyl cholesterol	32
24ß-Methyl cholesterol	75
Brassicasterol	100
Ergosterol	100
Lanosterol	< 1
24-Dihydrolanosterol	< 1
Cycloartenol	< 1

[a] The per cent growth is the cell count of Saccharomyces cerevisiae cells cultured under anaerobic conditions after 72 hr. (in sealed experimental vessels) divided by the count obtained with flasks supplemented with ergosterol. The values are averages of at least two experiments. Adapted from ref. 46.

that results in enzyme assays involving cycloartenol and lanosterol. Here, the difference in activity is thought to be due to the two molecules assuming very different shapes - bent or flat, respectively, when bound to the enzyme (*59*). We have recently challenged this view and introduced data that suggests enzymes that act on the sterol substrate bound and catalyzed these molecules in a precise planar geometric alignment, including cycloartenol and its cyclopropyl steroid cogeners (*60*). Our evidence that sterols which pass through the sterol pathway to completion exist in similar ground state conformations is based on the solution (*31-33*) and solid state (*30, 32* and Griffin, Wong and Nes unpublished data) properties of cyclopropysterols and related isomers which we have shown are flat with the side chain oriented in the "right-handed" conformation, like lanosterol and cholesterol. Two exceptions are 10α-cucurbitadienol (*27*) and euphol (*30*) which either exist in the bent conformation or with the side chain in the "left-handed" conformation.

Additionally, we have begun to use enzyme systems from plants. Thus, we have made a comparison of the K_m and V_{max} values for methylation of lanosterol and cycloartenol in the sunflower assay (*49, 60*). For this specific example, the two sterols possessed similar K_m values but different V_{max} values for methylation (*60*).

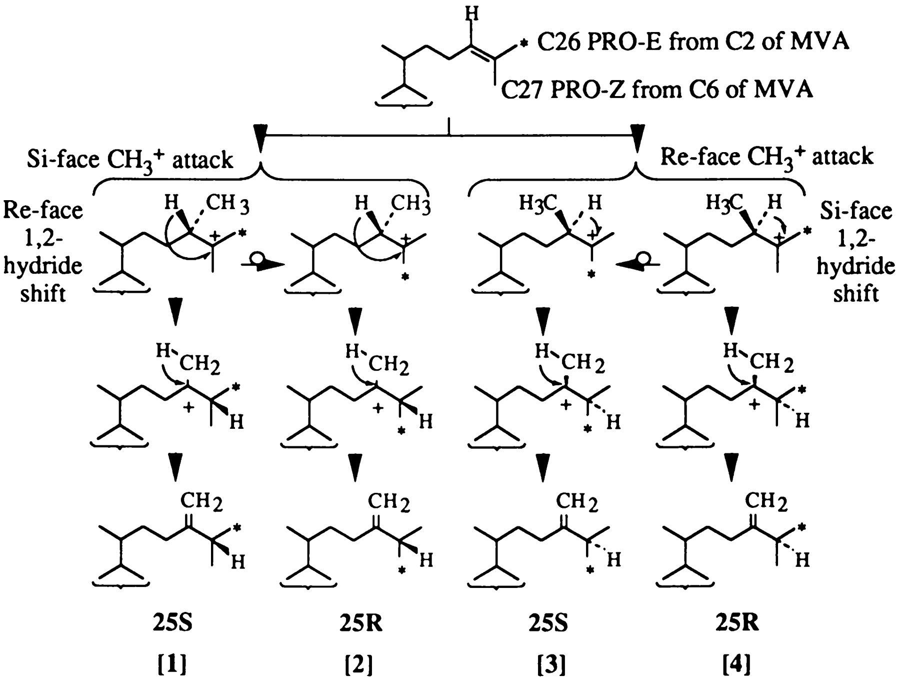

Fig. 11. Alternative mechanisms of C-24 methylation of the 24,25-double bond. Note the possible inversion mechanisms, routes 2 and 3.

The K_m and V_{max} are two major factors which together indicate catalytic competence. The difference in catalytic competence observed for lanosterol and cycloartenol has been thought to be due to their abilities to adopt into the flat and bent conformations (*59, 61*), respectively. If cycloartenol and other cyclopropyl sterols (31-nor cycloartenol and 24-dehydropollinastanol) were to assume the bent shape when bound in the active site, then its side chain relative to the side chain of lanosterol would be juxtaposed on the surface of the enzyme in a different region in space. On the assumption that only one ternary complex exists i.e., olefin, SAM and enzyme, for transmethylation, then for catalysis to proceed one of the two molecules must undergo conformational change. If the enzyme possesses a hydrophobic pocket acting as a springboard whereby cycloartenol flips from the bent to the flat conformation then a mechanism exists which would readily explain rate changes in the catalysis of lanosterol and cycloartenol (*60*). We determined to the contrary that the structural differences brought about by the nuclear shape of these two molecules are minimal and that the important structural features mediating catalysis were the conformation of the side chain and the tilt and polarity of the C-3 OH group (*60*). We have also found that subtle changes in the sterol conformation affect the chromatographic behavior of sterols in a predictable manner (*61*). Thus, knowledge gained about the chromatographic conformation may bear on the physiological conformation of sterols, as discussed elsewhere (*61*).

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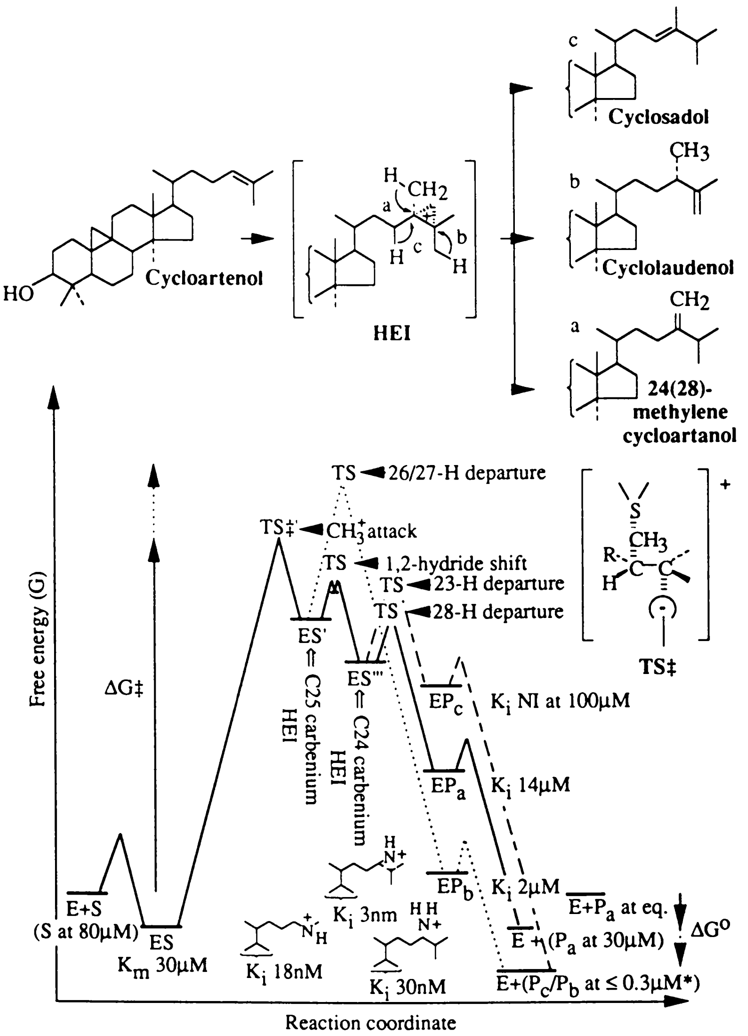
Cycloartenol
HO
HEI
H
CH2
a
b
c
H
H
c
Cyclosadol
CH3
b
Cyclolaudenol
CH2
a
24(28)-methylene cycloartanol
Free energy (G)
ΔG‡
TS 26/27-H departure
TS‡' CH3+ attack
TS 1,2-hydride shift
TS 23-H departure
TS' 28-H departure
ES'
C25 carbenium HEI
ES'''
C24 carbenium HEI
S
CH3
R
C
C
H
TS‡
EPc
Ki NI at 100μM
EPa
Ki 14μM
EPb
Ki 2μM
E+S
(S at 80μM)
ES
Km 30μM
Ki 18nM
Ki 3nm
Ki 30nM
H H N+
N+ H
E+Pa at eq.
E + (Pa at 30μM)
E+(Pc/Pb at ≤ 0.3μM*)
ΔG°
Reaction coordinate

Fig. 12. Hypothetical free-energy diagram for the alternative outcomes of C-24 methylation. A first approximation of the relative energies of the reaction course intermediates are as shown; ES, enzyme-substrate complex, TS, transition state and EP, enzyme-product complex. Relative energies of the intermediate species were deduced from studies of product inhibition and the use of compounds designed as high energy intermediate (HEI) analogues (Ref. 49, 58, 64). Endogenous microsomal concentrations are as indicated on the figure; S, substrate (cycloartenol), P_a, product (24(28)-methylenecycloartanol), P_b (cyclolaudenol), P_c (cyclosadol), where, *, 0.3μM represents our limit of detection by GC/HPLC. Microsomal preparations contained ca. 30μM endogenous cycloartenol. A typical assay was performed with 50μM cycloartenol, 50μM SAM and Tween 80 for 45 minutes at 30°C.

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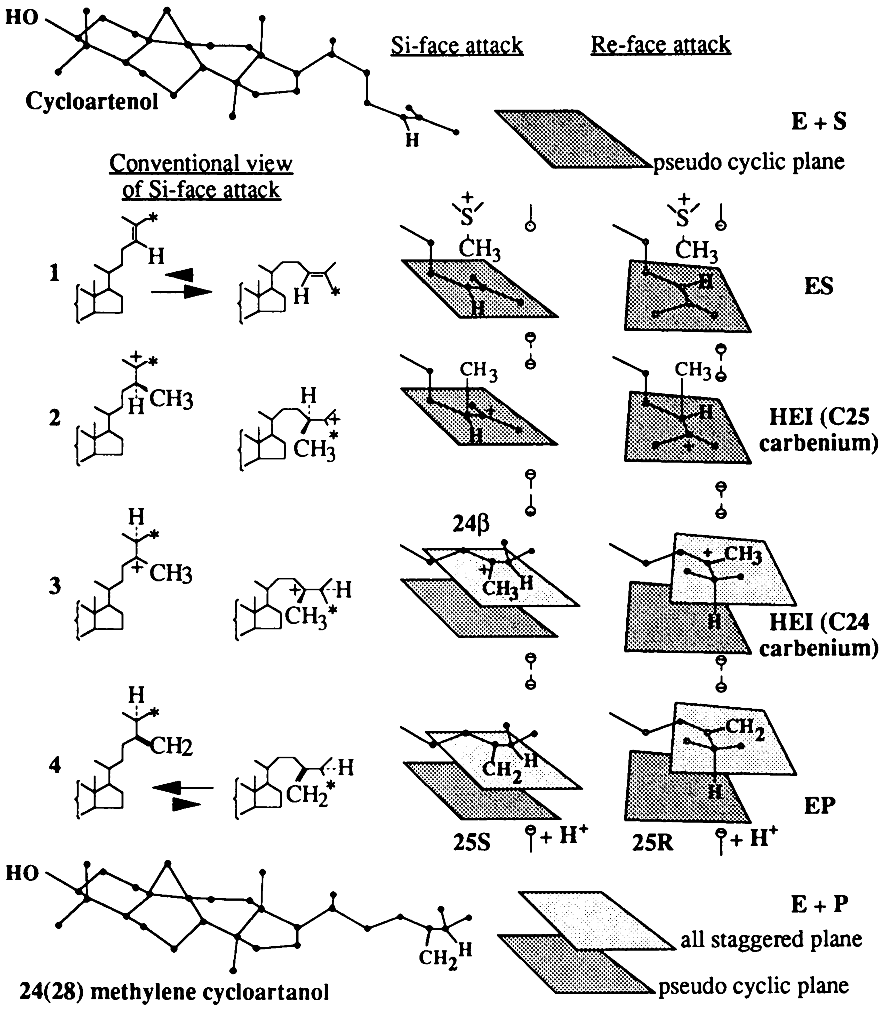
HO
Cycloartenol
H
Si-face attack
Re-face attack
E + S
pseudo cyclic plane
Conventional view
of Si-face attack
1
H
H
*
S
CH3
S
CH3
ES
2
H
CH3
H
CH3*
CH3
CH3
HEI (C25
carbenium)
3
H
CH3
H
CH3*
24β
CH3
H
CH3
HEI (C24
carbenium)
4
H
CH2
H
CH2*
CH2
H
CH2
H
EP
25S
+ H+
25R
+ H+
HO
CH2
H
E + P
all staggered plane
pseudo cyclic plane
24(28) methylene cycloartanol

Fig. 13. Hypothetical C-24 methylation mechanism in sunflower. The importance of side chain conformation to the methylation reaction is indicated in the diagram.

The importance for the side chain to orient into the pseudocyclic conformation in the ternary complex follows from two pieces of data: Firstly, the solid state conformation of lanosterol and cycloartenol indicate a natural proclivity for the side chain to deviate from the all staggered conformation into the pseudocyclic conformation. Secondly, enzymological data show that when the side chain is frozen into the all staggered conformation, e.g., as in cholesta-5,7,22,24-tetraenol the sterol is not a suitable substrate nor is such a sterol an effective inhibitor of the C-24 methyl transferase reaction; as evidenced by the K_i for sitosterol of 26 μM (*49*) and no inhibition of the reaction by stigmasterol at the highest concentration tested of 100 μM (*58*)). As shown in Figure 13, the reaction

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course is proposed to proceed via si-face attack of SAM on the Δ^{24} bond of cycloartenol with 1,2-hydride shift of H-24 to C-25 occurring from the re-face with no inversion of the terminal isopropyl group (*25*). The enzymatic reaction proceeds through several stages; the formation of a complex between enzyme and substrate, the conversion of this complex to an enzyme-transition state (TS)/high energy intermediate (HEI) complex, and the further conversion to an unstable complex between enzyme and product that can dissociate. The specific binding of substrate to enzyme therefore serves not only to confine particular reactions to a few compounds, but also to generate a stable complex that favors the spontaneous formation of a more reactive molecular shape. Here, the conformation of the side chain is a determinant in the reaction course. In the pseudocyclic conformation the double bond face may confront SAM which is located on the upper cleft of the enzyme or the ß-face relative to the sterol nucleus. This conformation is stable but nevertheless, potentially reactive; as such it contributes the driving force through conformational instability following formation of the HEI in the formation of a 24(28)-methylene sterol.

We now provide additional insight into the topology of the C-24 methyl transferase active site where significance is given to the nucleophilic center at C-3 upon the catalysis of the nucleophile at Δ^{24}. Several groups have studied the properties of the C-24 methyl transferase from a fungal (*62, 63*) and plant (*59*) assay system. They used a series of ground state and HEI analogues to demonstrate the importance of electrostatic interactions that evolve from methylation of the Δ^{24} bond. Our data shown in Figure 14 (*58, 64*) confirm the involvement of a transient interaction between the C-25 carbocation HEI and a nucleophile in the active site (*59*).

We have also prepared another set of putative HEI analogues to study the methylation reaction but with the purpose to demonstrate that the enzyme recognizes the molecule as a whole in order for catalysis to proceed with maximal efficiency. We considered the possibility that the enzyme possessed two strategic sites; one which recognized the proximal end of the sterol molecule - acting as a proton donor, the other at the distal end of the molecule which acts as a proton acceptor. Both sites play a role in catalysis: that is to affect binding and to mediate the molecular dipole through which methylation proceeds to completion. The molecular dipole which generates a charge distribution across the entire molecule is responsible for promoting the E-S formation. This is distinct from the dipole species that is generated in the ternary complex involving SAM, the Δ^{24} bond and the enzyme. We have also considered whether one or more nucleophiles (counterions) are present in the active site and their geometric relationship to SAM.

Our evidence that the enzyme maintains a specifically located basic site for the C-3ß OH moiety acting as a H-donor and is not simply a hydrophobic pocket which anchors the proximal end of the sterol through weak isosteric interactions is based on the following evidence. First, by incubating in the sunflower assay system a series of cyclopropyl sterols that differed in the degree of C-4 methylation-cycloartenol, 31-nor cycloartenol and 24-dehydropollinastanol (Fig. 15), we found

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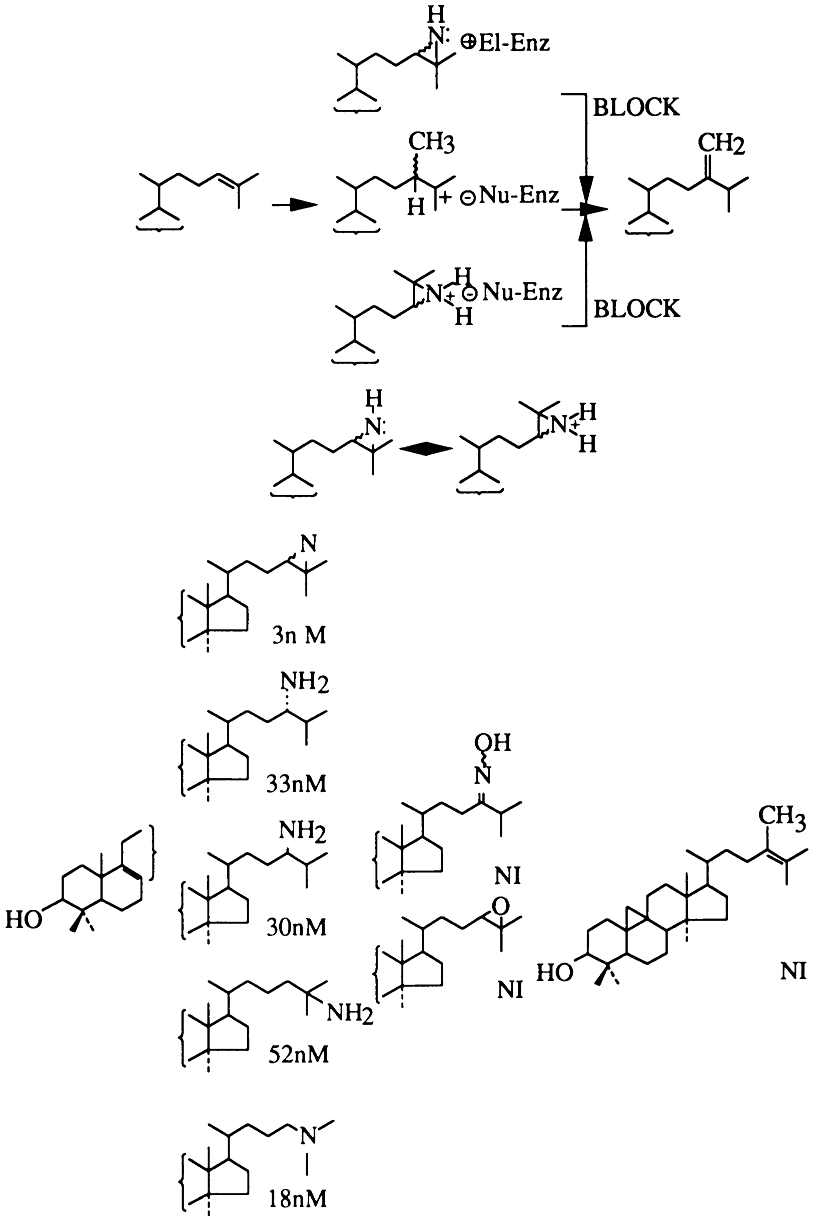

H
N: ⊕El-Enz
BLOCK
CH3
CH2
H
+ ⊖Nu-Enz
H
N+ ⊖Nu-Enz
H
BLOCK
H
N:
N+
H
H
N
3n M
NH2
33nM
NH2
30nM
NH2
52nM
N
18nM
HO
OH
N
NI
O
NI
CH3
HO
NI

Fig. 14. Hypothetical interaction of the C-25 carbeniun ion of the sterol with the nucleophile on the active site of the enzyme. The substrates shown were added together with a fixed concentration of cycloartenol to the sunflower assay as described elsewhere (*49*, *58*, *64*). NI, not inhibitory at the highest concentration tested.

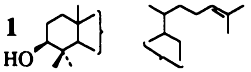
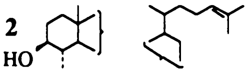
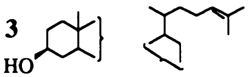
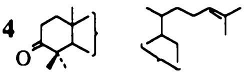
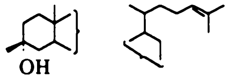
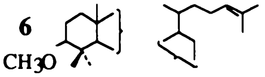
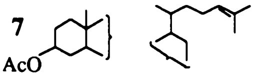
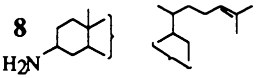
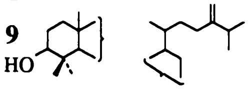
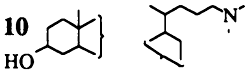
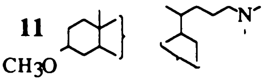
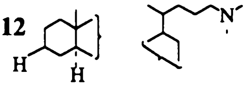
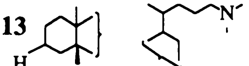
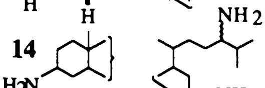
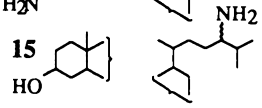
	SUBSTRATE	V_{MAX} (pmol/min)	K_M (µM)	%T	K_I
1		27.4 10.2	32 28	100 30	cycloartenol lanosterol
2		16.4	36	66	
3		7.4	43	32	
4		ND	ND -	28	
5		ND	ND -	0	
6		ND	ND -	0	
7		ND	ND -	1	
8		ND	ND -	0	500nM
9		ND	ND -	0	14µM
10		ND	ND -	0	24nM (25 aza cholesterol) 18nM (25 aza lanosterol)
11		ND	ND -	0	55nM
12		ND	ND -	0	45nM
13		ND	ND -	0	600nM
14		ND	ND -	0	250nM
15		ND	ND -	0	30nM

Fig. 15. Sterols tested as substrates and/or inhibitors of the C-24 methyl transferase of sunflower embryos (*49, 58, 64*). Note structure 14 represents 3β,24(R,S)-diaminolanosterol; see text for details. ND, not determined.

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that the three sterols possessed similar K_m values but the three sterols underwent different levels of transformation with the progression in activity from 0 to 1 to 2 methyls. This data, which demonstrated that the C-3 OH group is sensitive to steric hindrance from the neighboring C-4 grouping, suggests that catalytic competence is dependent on the interaction of the C-3 OH group with the enzyme. However, unclear was whether the OH group acted to donate or accept a proton so to facilitate general acid-base catalysis in a discrete region of the binding site. Examination of the activities resulting from 3-keto, 3-OAc, 3-OMe, and 3-amino derivatized sterols as substrates indicated that the C-3 OH on the natural substrate cycloartenol might be acting to donate electrons to bond other groups in the binding site (Fig. 15). The first three sterols retained a lone pair of electrons which may act as proton acceptors. Their impaired ability to act as substrates could be due either to the degree of tilt of the functional group away from the planar nucleus (toward the α-face, as for the 3-keto compound) or from steric bulk evolving from the added methyl group, as in the 3-OAc or 3-OMe compounds. The absence of transformation of the isosteric, but more basic, 3ß-amino sterol may be because of its inertness and possible protonation which may lead to electrostatic repulsion from the active site which in effect disturbs the free energy of the ensuing reaction.

For interaction, the side chain containing N-steroids are assumed to be protonated under physiological conditions and it is through nitrogen becoming a quaternary ammonium function (the salt form) that the ionic interactions proceed between the nucleophile on the enzyme and the charged N-steroid. By performing another experiment where the 3-amino sterol is used as an inhibitor of C-24 methylation we demonstrated that the 3-amino sterol was an effective inhibitor of the reaction. When the diamino sterol was used (3,24-diaminolanosterol) as an inhibitor, we found that the compound was intermediary in effectiveness between the corresponding monoamino sterols, 3-amino and 24-amino lanosterol (Fig. 15). Additionally, we observed that neither the polarity or conformation of the A-ring structure prevented the side chain containing N-sterol from binding and interfering with catalysis, since a series of structurally modified 25-aza sterols were good inhibitors. The degree of binding then is influenced by the extent of interaction between the C-3 OH group acting as a nucleophile and its potential to develop ionic character. The rate constants involved in kinetic control are determined by the conformation of the ring structure. Through conformational transmission effects developed in the nucleus both the tilt of the C-3 OH group and the side chain conformation are spatially modified (*60, 61*). Both structural features affect the overall free energy of the enzyme-substrate complex and subsequent catalysis. It follows that catalysis proceeds once the side chain enters favorably into the catalytic pocket. We have proposed a steric-electric plug model (Fig. 16) to illustrate the importance of the planar arrangement of the sterol where the C-18, C-19 and C-21 methyl groups fit specifically designated enzyme conduits. Additional proton withdrawing and proton donating groups exist within the active site, located at what are tantamount to either ends of the sterol molecule. Thus, a molecular dipole may be generated and individual dipoles in localized regions of the enzyme may also be generated to affect specific outcomes, e.g., with respect to the side chain metabolism. Two additional aspects of the model are the location of opposing

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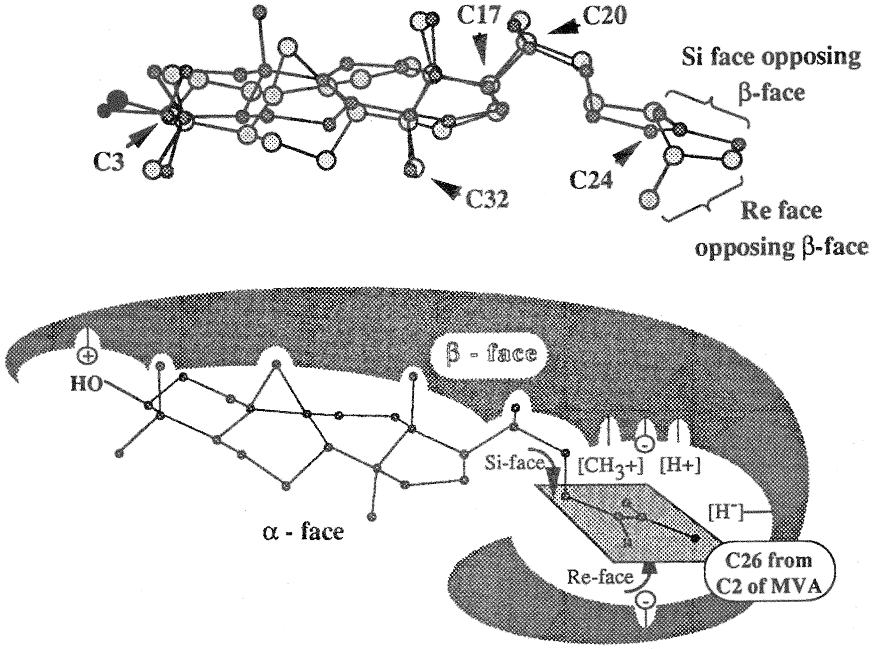
C17
C20
Si face opposing
β-face
C3
C32
C24
Re face
opposing β-face
β - face
HO
⊕
Si-face
[CH$_3$+] [H+]
[H$^-$]
α - face
Re-face
C26 from
C2 of MVA

Fig. 16. "Steric-electric plug" model of the binding and catalytic sites for metabolism of the Δ24-bond. The reactions hypothetically catalyzed on the same enzyme surface are C-24 methylation, reduction and isomerization. Superimposition of cycloartenol on lanosterol showing the Si and Re faces of the Δ24-bond.

In Regulation of Isopentenoid Metabolism; Nes, W., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1992.

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counterions toward the front of a canal into which the side chain may swing from a pseudocyclic to an all-staggered conformation and the placement of H^+ and NAD(P)H sites further back into the canal of the active site which may be used in the further metabolism of an olefinic bond i.e., isomerization and reduction. The function of the two counterions is for one (on the α-face of the molecule) to act as the nucleophile in the catalysis of the Δ^{24} bond, the other (on the ß-face of the molecule) to neutralize the positive charge on SAM. The positioning of the various groups are such to allow for an electrostatic bridge to be formed to trap the incoming Δ^{24} bond. The positions are based on the known or suspected stereochemistry at C-24 and C-25 (when chiral), of plant and fungal sterols which use SAM and NAD(P)H as cofactors in the metabolism of the Δ^{24} bond (*1, 25*).

One important feature of our model is that the usual order in which metabolism of the Δ^{24} bond proceeds in plants - methylation, a second methylation, isomerization and reduction is governed by the complementarity between substrate and enzyme, and cofactor availability. Specific modifications observed for a given substrate do not exclude the possibility that other outcomes are possible for different substrates but they are not the result of an enzyme with broad specificity (cf. ref. *64* for a key to the literature), for broad specificity and tight binding are not amenable to the model. Stereospecific outcomes are the result of a kinetically favored pathway. For a given substrate the rate constants for each of the metabolic events will have a fixed ratio. This may be determined by measuring the ratio of V_{max} values (assessed *in vitro*) or inferred from the actual concentration ratios (assessed by quantifying the sterol content *in vivo*) of plant sterols. Obviously when multiple incoming tetracycles enter into the sterol pathway, e.g., cycloartenol versus lanosterol or some other isomeric compound such as tirucallol, competition exists for the enzyme and the outcome may be influenced. Similarly, the rate of utilization of the outgoing sterol from the pathway (to the membrane) will influence discrimination between substrates and the observed character of the products.

Another feature then is the involvement of thermodynamic control where product utilization is proposed to promote the different kinetically favored pathways. For vascular plants the end product of metabolism of the Δ^{24} bond is sitosterol. Alternative sterols generated by the metabolism of the Δ^{24} may also be formed viz., cholesterol and campesterol. Whereas campesterol has never been found to be the major plant sterol, cholesterol may predominate the sterol mixture in plants with advanced age (*4*) or in specific cell-types, e.g., mesophyll cells (*4*). One possibility for the different compositions with growth and plant development may be the result of ontogenetically regulated enzymes, e.g., a C-24 methyl transferase and a 24,25-reductase, the levels of which increase and decrease, respectively with plant age, our first thought (*4*). In view of the steric-electric plug model that we have now proposed, in which the enzyme maintains multiple sites to carry out three reactions related to metabolism of Δ^{24} bonds, we suggest that the ontogenetically varied compositions are the result of thermodynamic control of the C-24 alkylation pathway. The shift from proceeding on with the second alkylation, to form sitosterol from a 24(28)-methylene sterol, to the isomerization of the 24(28)-methylene sterol, is a consequence of the relative positions of the equilibria for isomerization versus methylation and reduction. In order for the isomerization to

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proceed the other two reaction courses must be at equilibrium. The spontaneity for one or another side chain metabolism may be affected by the availability of appropriate sterol substrates and cofactors. For example, for reduction to follow methylation in plants we hypothesize that the preferred sterol structure used in methylations should be modified to produce a substrate which is more suitable for reduction, e.g., as in the conversion of cycloartenol to 24-ethyl desmosterol, and the level of SAM bound to the enzyme should be decreased whereas the level of NAD(P)H should be increased. It follows that the preferred reaction outcome is dependent on the global structure of the sterol and attendant cofactors bound to the enzyme and on the ability of the product to dissociate from the ES-complex and enter into other cellular compartments (membrane). The latter event will be influenced by prevailing physiological conditions, e.g., membrane biogenesis and C-3 derivatization to esters and glycosides.

Kinetically Favored Pathways and Their Role in Evolution. In order to discuss the phylogenetic significance for the operation of kinetically favored pathways, a balance sheet for the stoichiometry of sterol biosynthesis should be provided. The major biosynthetic requirements for sterol transformation involve the sterol substrate, SAM, NAD(P)H and ATP equivalents (Fig. 17 and Table 2). The cyclization of squalene oxide to sterols is an anaerobic process which does not seem to require cofactors (*65*). The C-24 alkylation pathway involves all the factors listed in Table 2. The formation of C-24 alkyl sterols is more energy expensive than the formation of cholesterol, in terms of ATP equivalents. Alternatively, there is no apparent difference in stoichiometry between C-24 epimeric sterols, even though their detailed C-24 alkylation pathways are very different. Nevertheless, stoichiometric regulation of the C-24 alkylation pathway may be the key to the alternative C-24 methyl and ethyl pathways in nature.

Next is examined by what is meant by regulation of "sterol" production. Here, the regulatory events that lead to the formation of the first tetracycle formed by squalene oxide cyclization will be distinguished from the regulatory events of further tetracycle transformations. Clearly, the regulatory strictures that govern the isopentenoid steps (pre squalene oxide) early in the sterol pathway will be different from the ones that mediate the post-cycloartenol (or lanosterol) steps. In our opinion, the correlation in enzyme activity (e.g., HMG-CoA reductase) with end product formation - ergosterol in fungi (*66, 67*) and sitosterol in plants (*7, 68*), particularly as it only relates to growth dynamics (*7, 66*), does not sufficiently warrant a determination of a rate-determining step.

For an enzyme to be rate-determining of 24-alkyl sterol sterol production i.e., involving only those enzymes that act on the sterol substrate, the following rules should be considered: (1) The activity of the enzymes should be controlled by the substrate specificity and attendant cofactor requirements both of which are determined by the mechanism of the reaction sequence rather than simply the level of sterol that passes through the pathway, (2) the regulatory step should involve an enzymatic catalysis in which the concentrations of substrates and products involved in the catalysis are far from thermodynamic equilibrium, (3) the maximal velocity of the regulatory enzyme, as measured in cell-free extracts under optimal

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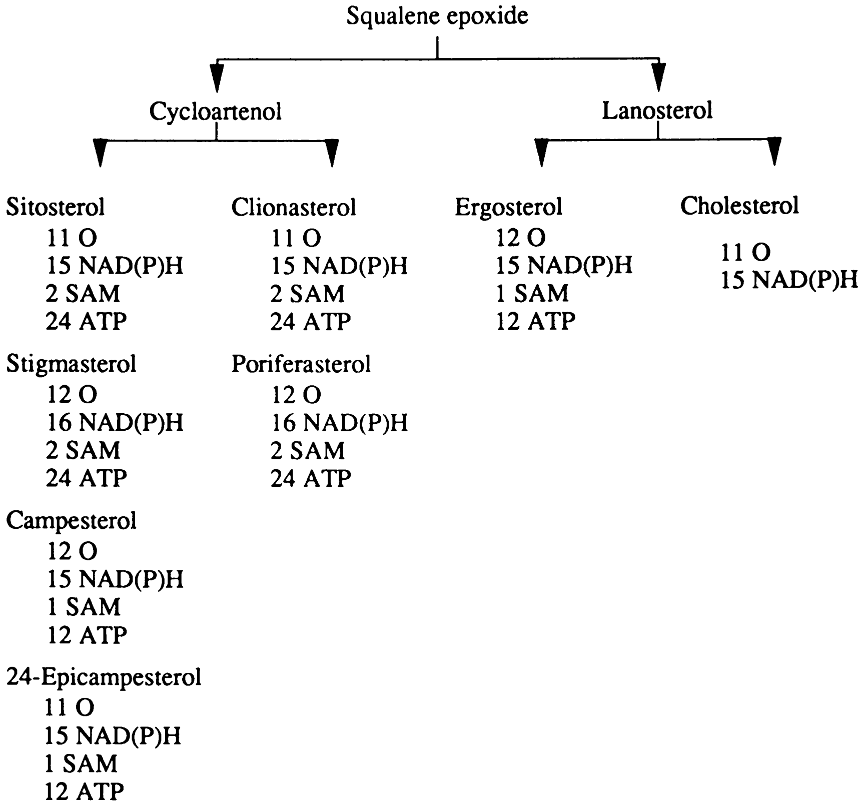
Squalene epoxide
Cycloartenol
Lanosterol
Sitosterol
11 O
15 NAD(P)H
2 SAM
24 ATP
Stigmasterol
12 O
16 NAD(P)H
2 SAM
24 ATP
Campesterol
12 O
15 NAD(P)H
1 SAM
12 ATP
24-Epicampesterol
11 O
15 NAD(P)H
1 SAM
12 ATP
Clionasterol
11 O
15 NAD(P)H
2 SAM
24 ATP
Poriferasterol
12 O
16 NAD(P)H
2 SAM
24 ATP
Ergosterol
12 O
15 NAD(P)H
1 SAM
12 ATP
Cholesterol
11 O
15 NAD(P)H

Fig. 17. Stoichiometry in sterol biosynthesis.

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Table 2. Cofactor Requirement and Consumption in Sterol Biosynthesis

Reaction Step	*NAD(P)H*	*Oxygen*	*SAM*	*ATP*[1]
C-14 Demethylation[2]	3	4	-	-
C-4 Demethylation (first)	4	3	-	-
C-4 Demethylation (second)	4	3	-	-
$\Delta^{14(15)}$ Reduction	1	-	-	-
$\Delta^{24(25)}$ Reduction	1	-	-	-
$\Delta^{7(8)}$ Reduction	1	-	-	-
$\Delta^{5(6)}$ Dehydrogenation	1	1	-	-
$\Delta^{24(28)}$ Reduction	1	-	-	-
$\Delta^{25(27)}$ Reduction	1	-	-	-
$\Delta^{22(23)}$ Dehydrogenation	1	1	-	-
$\Delta^{24(25)}$ Methylation	-	-	1	12
$\Delta^{24(28)}$ Methylation	-	-	1	12

[1] Number of ATP equivalents thought to be used in C-24 methylation (L. W. Parks, personal communication).

[2] This step is assumed to undergo C-14 demethylation to form the 8,14-diene sterol.

conditions, should be one of the slower enzymes in the pathway and (4) the concentration of the natural substrate for the regulatory step should increase faster than its ability to be modified by other enzymes when the regulatory enzyme is interfered with by changes in substrate (including blocks created by sterol biosynthesis inhibitors that mimic the HEI) or cofactors.

Two consequences of these rules are that the intermediate that accumulates due to the defective regulatory step should be harmful to cellular physiology and that the end product(s) should be functionally superior to their precursor molecules. Additionally, the end products should be capable of feed back regulation by binding with the regulatory enzyme and interfere with its catalysis, i.e., become an allosteric effector.

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Examination of the enzymological literature indicates, for wild-type yeast cells (*69-79*), there may be a kinetically favored pathway which leads directly to one end product - ergosterol, during active cell proliferation with regulation of the C-4 demethylation step representing the rate limiting step. A second favored pathway may become operational at growth arrest that uses ergosterol as substrate (to produce ergosta-5,7,9(11),22-tetraenol and other oxidized ergostenols such as ergosterol endoperoxide (*39*)). The pathway given in Figure 18 for the biosynthesis of fungal ergosterol differs from other pathways which show the transformation of lanosterol to ergosterol involves a matrix of crisscrossing pathways that converge into ergosterol with no specific control point in the post-lanosterol pathway (*80, 81*).

That C-4 demethylation is the rate-determining step follows from the observations that show of the enzymes tested in cell-free preparations, the C-4 demethylase(s) prefers the tetracycle which naturally enters the pathway first (lanosterol), it is one of the slower acting enzymes in the pathway, and the rate of C-4 demethylation acts as a physiological determinant in the production of impaired growth. The latter view follows from the structure-activity studies performed with yeast (*46,82*) in which C-4 demethylation was an absolute requirement for architectural utilization of sterols. In sunflower plants the C-24 alkylation pathway may be rate-limiting for sitosterol production (*49*). At what point will the kinetically favored pathway shift to the synthesis of other sterols? One mechanism to alter the kinetics is to disturb the equilibria (thermodynamic control) for the various enzymes. This may be brought about by changes in the physiological conditions (aerobic versus anaerobic/light versus dark) which affect cofactor supply, adding sterol biosynthesis inhibitors to the culture medium, or mutations which interfere with the synthesis of some step in the pathway. All of these changes ultimately impact on the sorts of sterols that will be formed during the course of metabolism. Assuming the rate constants for each of the several steps in lanosterol transformation to ergosterol are influenced by the global molecular shape of the sterol and its complimentarity with the enzyme then the rate of product formation at each step and the final product will be dependent on the prevailing physiological conditions. Thus, by extrapolating from the yeast pathway, the variety of sterols which occur in nature can be accounted for by the operation of a select few enzymes. Each cell and organism is assumed to have evolved their own kinetically favored pathway. Kinetically less favored pathways may be introduced into the

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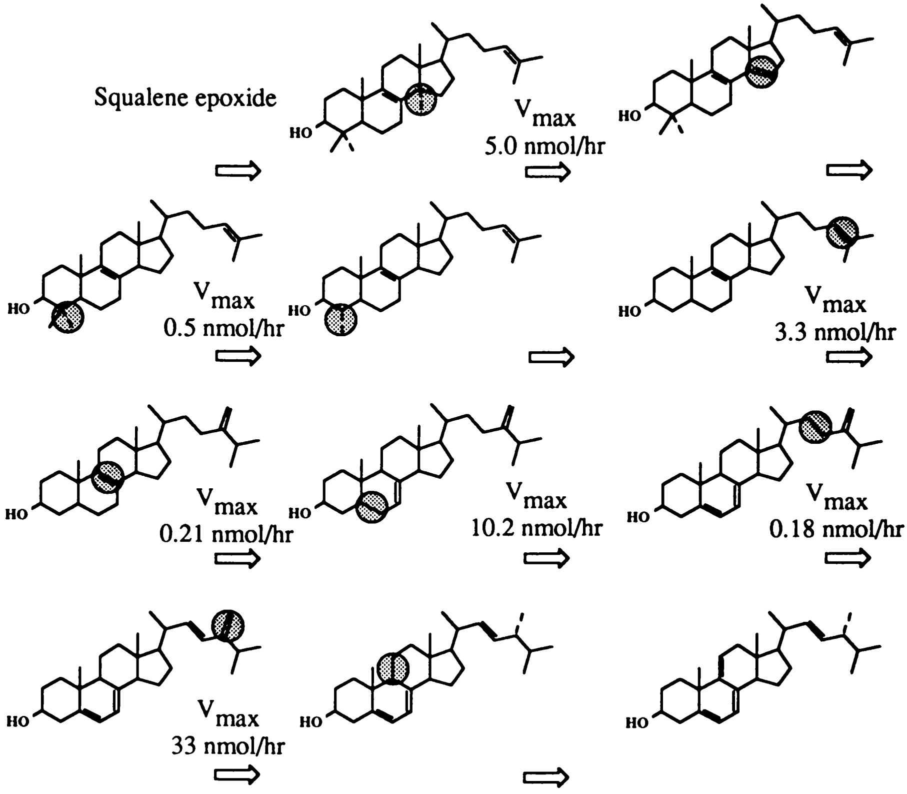
Squalene epoxide
HO
Vmax
5.0 nmol/hr
HO
HO
Vmax
0.5 nmol/hr
HO
HO
Vmax
3.3 nmol/hr
HO
Vmax
0.21 nmol/hr
HO
Vmax
10.2 nmol/hr
HO
Vmax
0.18 nmol/hr
HO
Vmax
33 nmol/hr
HO
HO

Fig. 18. Hypothetical kinetically favored pathway for the biosynthesis of ergosterol in yeast.

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biosynthetic scheme of an organism after a shift in the stoichiometry of cofactors, usually resulting at the onset of growth arrest.

Even though all three outcomes of C-24 methylation may proceed on the same enzyme surface, the C-24 methyl transferase may undergo an evolutionary transition from one using a lower energy route (primitive) to one using a higher energy route (advanced) involving proton loss versus hydride shift. This evolution may proceed within the context of kinetically favored pathways: the increase in biosynthetic choices that prevail in forming C-24 ethyl sterols from a 24(28)-methylene sterol compared with the choices that prevail from a 23(24)- or 25(27)-sterol. The choices are coupled to the utilization of the products (thermodynamic control) as membrane components where the increased bulk at C-24 should increase the molecules fitness as a membrane insert (*4*).

The cycloartenol-lanosterol bifurcation seemingly contradicts our proposal. Here, there is assumed an absolute block in the pathway with respect to the 9ß,19-cyclopropyl to Δ^8 isomerase (*2, 83-87*). We considered earlier that absolute blocks might also be operating for other steps in the pathway in plants and this difference in genealogy might account for the different sterol compositions (*2, 13*). It is generally thought that non-photosynthetic organisms that cyclize squalene oxide to lanosterol should not be capable of opening the 9ß,19-cyclopropyl ring in cyclopropyl sterols. Investigations with cell-free systems obtained from yeast (*86*) and other non-photosynthetic systems (animals) (*87*) have shown that cycloartenol fails to undergo conversion to lanosterol, whereas in photosynthetic plants, the cyclopropyl group is readily isomerized into the Δ^8 position (*2*). Cycloartenol may also give rise to parkeol, the 9(11)-isomer of lanosterol (*88, 89*). We have recently reexamined whether cycloartenol may undergo conversion to ergosterol. When wild-type yeast cells (whole organism) are incubated with 2-tritio cyclartenol or 2-tritiolanosterol, only the 2-tritio lanosterol was converted to ergosterol (Xu and Nes, unpublished). Barton and coworkers also tested whether 2-tritio parkeol could be converted to ergosterol by wild-type yeast cells, which it was not (*90*). The enzymological data was confirmatory for the whole cell feedings with labeled substrates that cycloartenol was not converted to ergosterol by yeast. We now have new evidence that yeast, the sterol mutant GL7, will convert cycloartenol to ergosterol via a 9(11)-sterol intermediate (*39*). The rationale for our new findings with the mutant is discussed in light of the kinetically favored sterol pathway that yeast operate.

The sterol mutant GL7 is auxotrophic for sterol due to a defective squalene oxide to lanosterol cyclase and a lesion in the heme pathway (*91*). The sterol mutant fails to synthesize significant levels of membrane ergosterol de novo (although it is leaky producing hormone levels of sparking ergosterol (*92*)) due to the block with squalene oxide cyclization. The mutant therefore cannot produce significant levels of lanosterol which may compete with the dietary sterol for yeast enzymes that act on the sterol substrate. We have developed culture conditions that amplify heme-competence but retain a no growth response in the absence of dietary sterol. We refer to these cells as sterol-adapted cells (*82*). Lanosterol incubated with the cells is converted to ergosterol in high yield (*93*). Ergosterol fed to the cells is converted to ergosta-5,7,9(11),22-tetraenol in low yield.

We had previously observed that ergosterol was converted to the tetraene in wild-type yeast and other fungi at the onset of growth arrest (*39*). We considered a 9(11)-dehydrogenase was induced late in the growth cycle. When cycloartenol and parkeol were fed to the sterol-adapted GL7 cells both were converted to ergosterol (*93*). In contrast to the lanosterol feed where intermediates did not accumulate, in the cycloartenol and parkeol feeds the cells accumulated 4,4-dimethyl and 4-monomethyl sterols. In the cycloartenol incubation only 31-nor cycloartenol was detected in the cells. However, in the 4,4-desmethyl sterol fraction the sterol composition was a mixture of ergosterol, 14α-methyl ergost-9(11),24(28)-dienol, and a series of 24 alkyl pollinastanols (*93*). Thus, the C-24 methyl transferase from yeast can methylate cyclopropyl sterols. This finding suggests that the methyl transferase from yeast is not that different from plants, only that it maintains a slightly different substrate requirement to ensure the reaction outcome which leads preferentially to the 24(28)-methylene sterol. Because 24-dehydropollinstanol (a cycloartenol cogener with the geminal C-4 methyls removed) conformationally mimics zymosterol, the preferred substrate of the reaction in yeast, it was not surprising that a cyclopropyl sterol might serve as a sterol source for transmethylation. The inability for cycloartenol to act as a substrate for transmethylation by yeast is apparently due to steric repulsion by the C-4 methyl groups; not from the putative bent structure of the ring system, as indicated by others (*35, 59*). 24-Dehydropollinastanol also mimics the structure of ergosterol. Therefore, it may have served as the substrate for ring opening. In the conversion of ergosterol to ergosta-5,7,9(11),22-tetraenol and 5α,8α-epidioxyergosta-6,22-dienol (ergosterol endoperoxide), lanosterol to lanosta-7,24-dienol and cycloartenol to parkeol, lanosterol or 10α-cucurbitadienol there involves a protonation-deprotonation process to initiate the three mechanistically distinct outcomes shown in Figure 19:9(11)-dehydrogenation, 8 to 7 isomerization and the cyclopropyl ring to Δ^8-isomerization. These varied events may proceed on the same enzyme surface. This requires a residue in the active site within the vicinity of the B/C ring junction that may act as proton donor or acceptor. The pK_a of this catalytic residue will in turn be dependent upon its environment and upon substrate association/dissociation. The catalysis demonstrated by the active site will be influenced by the bound substrate. Therefore, this enzyme (or homologues) like the "methyl transferase" may be responsible for the catalysis of multiple yet distinct conversions for which the C-8-9-10 carbenium ion is a common intermediate.

From these considerations of catalytic mechanisms for the multiple and varied conversions that occur in sterol biosynthesis, we speculate that a continuum of catalytic activities exists across the evolutionary hierarchy. The diversity observed in sterol occurrence is a consequence of the kinetic and thermodynamic control principles discussed above. This is a reflection of the ultimate product(s) which are required to satisfy, qualitatively and quantitatively, the cellular requirements throughout the life history and evolution of the organism.

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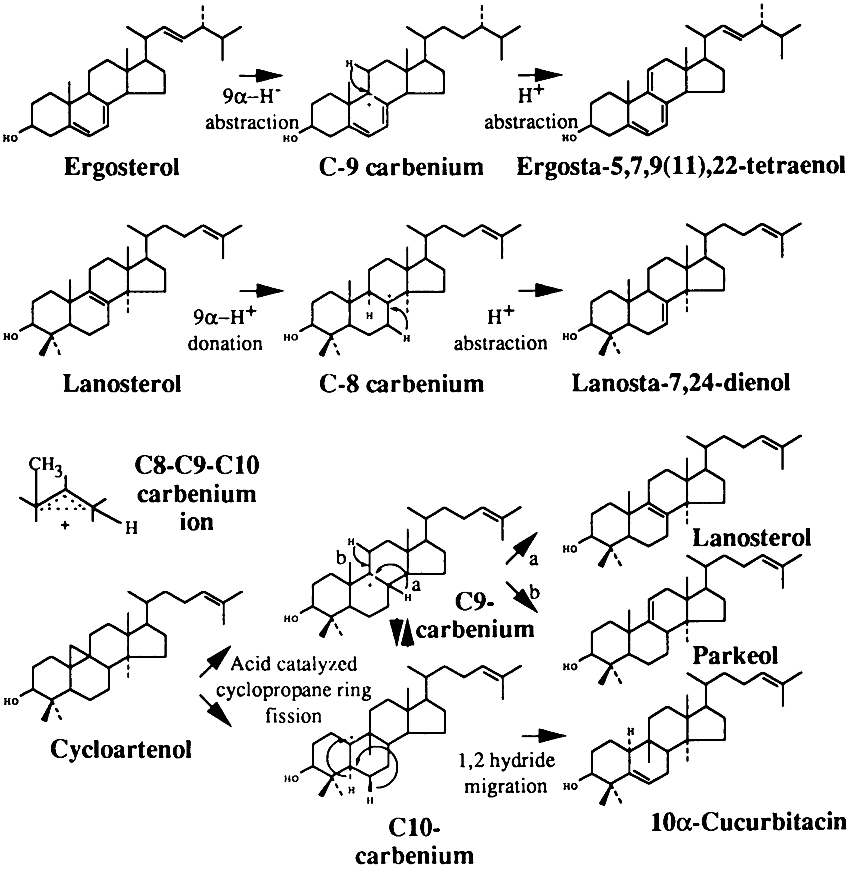
9α-H⁻
abstraction
H⁺
abstraction
Ergosterol
C-9 carbenium
Ergosta-5,7,9(11),22-tetraenol
9α-H⁺
donation
H⁺
abstraction
Lanosterol
C-8 carbenium
Lanosta-7,24-dienol
CH3
C8-C9-C10
carbenium
ion
H
b
a
C9-
carbenium
a
b
Lanosterol
Parkeol
Acid catalyzed
cyclopropane ring
fission
Cycloartenol
1,2 hydride
migration
C10-
carbenium
10α-Cucurbitacin
HO
H

Fig. 19. Similarities in the formation of a central C8-C9-C10 carbenium ion by the sterols shown in the figure.

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Chapter 11

Biosynthesis of Oxysterols in Plants, Animals, and Microorganisms

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As a class of compounds, oxysterols have demonstrated a wide variety of important biological properties. The specific inhibition of sterol biosynthesis is of special interest since it may prove useful in the prevention of reversal of certain cardiovascular diseases and may also prove to be useful in the control of normal and abnormal cell growth. Under certain conditions biological systems have been induced to produce oxysterols which adds support to the hypothesis that oxysterols may be natural regulators of sterol biosynthesis in the intact cell. Little attention has been directed to the occurrance and biosynthesis of oxysterols in the total biosphere. In this report, we have focused on the evolutionary origins of these biosynthetic pathways which have been reported to occur in plants, animals, and microorganisms.

Steroids containing multiple oxygen functionality are widely distributed in nature. As a class of compounds, oxysterols can be defined as sterols bearing a second oxygen function, in addition to that of carbon-3, and having an iso-octyl or modified iso-octyl side chain (1-5). These compounds have demonstrated a variety of diverse biological properties, which include cytotoxicity, atherogenicity, carcinogenicity, mutagenicity, hypocholesterolemia, and effects on specific enzymes (1-11). They have been found in animal tissues and food stuffs (9) and have been isolated from drugs used in folk medicine for the treatment of cancer (12-14). Other studies have shown that certain oxysterols have demonstrated significant activity in the inhibition of DNA synthesis in cultured cells (15,16). A number of oxygenated derivatives of cholesterol and sterol intermediates in cholesterol biosynthesis have been found to be potent inhibitors of

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sterol biosynthesis in animal cells in culture (10,11,17). The specific inhibition of cholesterol biosynthesis in mammalian cells by oxygenated derivatives of cholesterol and lanosterol has been shown in many cases to decrease cellular levels of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity.

This reported inhibitor response has been attributed to a decreased rate of HMG-CoA reductase synthesis (17-19) and in some instances to an increase in enzyme degradation (17,19). Other oxysterols are known to depress the rate of cholesterol synthesis from lanosterol in rat liver homogenates and may inhibit the 14-demethylation of lanosterol (20-22).

An important property of many oxysterols is their ability to repress HMG-CoA reductase activity in cultured mammalian cells (9,10,22). This suppression of activity has been found to vary over a wide range depending on the structural features of the oxysterol. As a general trend, inhibitory activity increases as the distance between carbon-3 and the second oxygen group becomes greater. Sterols with an additional oxygen function in ring D and the side chain have been shown to have the greatest activity. An intact side chain is a requirement for potent activity; a decrease in the length of the (iso-octyl) side chain results in decreased activity (23). Other noticeable trends indicate a relationship between inhibitory activity and the extent to which the second oxygen function is sterically hindered. In general, axial hydroxyl groups are more hindered and possess lower activities than the less hindered equatorial conformation (10,23,24,). Steric hindrance from other parts of the steroid molecule also can result in diminished activity (i.e., effect of carbon-14 alkyl substituents on the carbon-15 hydroxyl group) (25). It has been suggested that oxygen functions in conformationally flexible positions, such as those in ring D and in the side chain, produce more inhibitory steroids due to increased effectiveness of hydrogen bonding or hydrophilic interactions with receptor molecules (11).

The experimental evidence described above suggests a regulatory mechanism which, by analogy to steroid hormone receptors and bacterial induction-repression systems, requires a binding protein to recognize oxysterols and mediate subsequent cellular events. There is evidence for the existence of a specific cytosolic receptor protein for oxysterols (23,26). After the activities of a number of sterols were evaluated, a good correlation was found between the actions of certain oxysterols on HMG-CoA reductase in L cells and their affinity for an oxysterol binding protein (23). Moreover, the actions of oxysterols which depress the rate of cholesterol biosynthesis from lanosterol and possibly inhibit the 14-demethylation of lanosterol are also postulated to exert their actions by an oxysterol binding protein (20-22).

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Discussion

This report discusses oxysterols resulting from primary metabolism. These will include sterols with an additional oxygen function at either the C-7, C-22, C-24, C-25, or C-32 carbon atom of the sterol nucleus and side chain (Figure 1).

It is well known that mammalian systems produce oxysterols. Derivatives of cholesterol hydroxylated in the 7α-, or 25-, or 26- positions are produced in liver during bile acid biosynthesis and in side-chain hydroxylation at the 2α- and 22R-positions in the initial step in the conversion of cholesterol to steroid hormones in endocrine organs (27-29). In addition, all cells produce 32-hydroxylanosterol during the conversion of lanosterol to cholesterol (30,31). The oxygenated intermediates lanost-8-en-3ß,32-diol and 3ß-hydroxylanost-8-en-32-al have been isolated from rat liver microsomes incubated with 24,25-dihydrolanosterol (32). A number of C-32 hydroxylated derivatives of cholesterol and lanosterol were shown to be potent inhibitors of HMG-CoA reductase, sterol biosynthesis, and possess a high affinity for the oxysterol binding protein in mammalian systems (23,33,34). Another mode of oxysterol biosynthesis has been described which utilizes the isopentenoid pathway to produce side-chain oxygenated derivatives of cholesterol and lanosterol (34,35). Such compounds are derived from squalene 2,3-epoxide by the introduction of a second oxygen function to form squalene 2,3;22,23-dioxidosqualene prior to cyclization. Thus, this intermediate has been shown to form 24(S),25-epoxylanosterol, 24(S),25-epoxycholesterol, and 25-hydroxycholesterol in mammalian systems (35-38). 24(S),25-Epoxycholesterol has been isolated from cultured mouse L cells, Chinese hamster lung fibroblasts, and human liver (39). These oxygenated side-chain derivatives have been shown to be potent inhibitors of HMG-CoA reductase, sterol biosynthesis, and possess a high affinity for the oxysterol binding protein (35-39). These results add support to the hypothesis that oxysterols may be natural regulators of cholesterol biosynthesis in mammalian cells (5).

The biosynthesis of sterols seems to be a ubiquitous property of all vertebrate animals and photosynthetic plants from the prokaryotic blue-green algae to climax angiosperms (40-46). In addition, biosynthesis also occurs in most fungi and some protozoa (44). Not enough attention has been given to the biosynthesis of oxysterols in plants and microorganisms. The goal of the present review is to demonstrate the evolutionary basis of the oxysterol biosynthetic pathways which have been discovered in mammalian models. A variety of oxygenated sterols are known to exist in and have been isolated from plants and microorganisms; these may be precursors to the sterols required for growth and/or reproduction or may be secondary plant metabolites.

Many of the most frequently encountered oxysterols are those with a keto or hydroxyl function at the C-7 position. Sterols oxygenated at C-7 and containing a Δ^5 double bond are among the most commonly found products of autoxidation (9). Sterols oxygenated at C-7 have been found in animal tissues and in foodstuffs and may play a role as physiological regulators of HMG-CoA reductase, sterol synthesis, and cell replication in the cells in which they are found (6,9,10,47,48).

Hydroxylation at C-7 (7α-hydroxylation) on the steroid nucleus appears to be the rate-determining step in the biosynthesis of bile acids from cholesterol. Also, it is the first step in the route leading to cholest-5-en-3ß,7α-diol in bile acid synthesis. 7α-Hydroxylase, a mixed-function oxidase requiring NADPH and molecular oxygen, has been recovered from the microsomal fraction of rat liver (44 and references therein).

The oxysterol cholest-5-ene-3ß,7ß-diol has been isolated from the putative Chinese drug Bombyx cum Botryte, and its toxic effect on hepatoma cells has been reported (12). This sterol and its water-soluble bishimisuccinate sodium salt have demonstrated preferential toxicity on various types of cancerous and proliferating cells in vitro (49) and in vivo (50). A number of other 7-oxygenated sterols have also been evaluated for their ability to inhibit tumor cell growth (51).

The 7-ketosterol 3ß-hydroxycholest-5-en-7-one has been reported to occur in sponge (52), the roots of Glossostelma carsoni (53), in Gonyaulax polygama (54), and in the Chinese drug Bombyx cum Botryte (55). Since this oxysterol is a common autoxidation product of cholesterol (9,56) its reported occurrence may, in some instances, be the result of unrecognized autoxidation rather than biosynthesis. 24-Alkyl-3ß,7-diols have been found in the sponge Haliclona oculata (57) and C_{28}-C_{29} 3ß, 7-diols are known to occur in the dried roots of the Chinese drug Euphorbia fischeriana (58). Ergostane type steroids, isolated from the stem and roots of Petunia hybrida, have been found to contain 7α-oxygenated functionality (59,60).

Several biotransformations brought about by plant tissue cultures have been reviewed (61). Cholesterol was oxidized by cultures of Euonymous europaca and Digitalis purpurea to produce 7-keto- and 7-hydroxy-derivatives (62) similar transformations of both cholesterol and fucosterol occurred with cultures of Coriolus hirstus (63). Oomycetes are known to produce Δ^5-7-keto sterols from Δ^5-sterols during the last steps in the biosynthesis of antheridiol and oogoniol (64). Demethylation of the C-14 methyl group (C-32 of lanosterol) occurs at the aldehyde stage of oxidation (32,64-66). Formic acid results from incubations of [32-^{3}H] lanost-7-en-3ß,32-diol with liver

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microsomes under aerobic conditions in the presence of a NADPH generator (66). In the absence of NADPH (generator), the 32-formyl steroid could be recovered unchanged. 4,4-Dimethylcholesta-7,14-dien-3ß-ol was the only steroid product recovered from the demethylation incubations.

In more recent studies, 3ß-hydroxylanost-8-en-32-al has been isolated from incubations of radiolabelled 24,25-dihydrolanosterol with rat liver microsomal preparations (32, 67). A nuclear double bond seems to be required for 14α-lanostanol since lanosterol was recovered without apparent transformation when incubated with rat hepatoma cells (68).

During the biosynthesis of sterols in yeast, 14α-demethylation is proposed to proceed in the same manner as described for animal tissues (69,70). Cytochrome P-450 catalyzes demethylation of the methyl group at C-14 of lanosterol. Yeast mutants, blocked in demethylating C-32 of lanosterol, were shown to possess low levels of P-450 (71). In fungi, 14α-demethylation occurs in an analogous manner (72) and in plants this process (the 14α-demethylation of cycloartenol metabolites) apparently proceeds in a similar manner (44 and references therein).

In adrenal glands, 20α, 22R-dihydroxycholesterol as well as the 20α - and 22R-monohydroxycholesterols have been found to occur (44). Cytochrome P-450 catalyzes the oxidative side chain cleavage of cholesterol to yield pregnenolone, the precursor of a variety of steroid hormones in both the adrenyl cortex and corpus luteum. The side chain cleavage enzyme normally catalyzes a triple hydroxylation of cholesterol, beginning with hydroxylation at position 22R (73,74). The cholesterol side chain cleavage reaction occurs via a NADPH/O_2-dependent three-step oxidation: the first hydroxylation appears to occur at position 22R, the second at position 20α and a third oxidation results in cleavage of the 20-22 carbon-carbon bond, thus producing 22R-hydrocholesterol and 20α, 22R-dihydroxycholesterol as enzyme bound intermediates of the side chain cleavage reaction. The initial hydroxylation of C-22 requires the abstraction of hydrogen from the position to be hydroxylated. Cholesterol and also 22R and 22S-hydrocholesterol (both of which contain an abstractable hydrogen at C-22) are readily metabolized by cytochrome P-450 to pregnenolone.

Sterols containing an oxygen function at C-22 have demonstrated diverse biological properties. 3ß-Hydroxycholest-5-en-22-one, 22R- and 22S-hydroxycholesterol have demonstrated one or more of the following properties: inhibition in cultured cells of sterol biosynthesis from acetate; inhibition of HMG-CoA reductase; affinity for an oxysterol binding protein; and inhibition of the P-450 oxidation of the cholesterol side chain at C-22 (22-

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ketocholesterol) (23,74,75,78). These results indicate their importance as potential metabolic bioregulators.

As general topics, the evolution of lipids (79) and of endocrine systems (80) have been extensively investigated. These studies indicate the existence of proteins that bind mammalian sex steroids in a number of primitive species, including fungi and unicellular eukaryotes (81). Other studies have demonstrated the existence of mammalian sex steroids in yeast and higher plants (82-87). These results demonstrate the presence of enzyme systems, in these organisms, which are capable of degrading the side chain of steroids. The literature contains many examples of 22-oxygenated steroids which have been isolated from plants and microorganisms (88-93). It is therefore possible that higher organisms are the recipients of metabolic pathways, which produce C-22 oxygenated sterols, that originated in more primitive species.

From a biosynthetic perspective, the most interesting pathway, from which oxysterols are derived, is that which involves the cyclization of 2,3;22,23-dioxidosqualene (DOS) to form sterols oxygenated at C-24 and 25. Enzymatic conversions of some terminally modified squalene 2,3-oxide analogs into the corresponding lanosterol analogs have shown that the substrate specificity of the enzyme is low (94-97). A quantitative comparison of the relative efficiency of transformation of squalene 2,3-oxide and its terminally modified analogs (including DOS) was studied using the cyclase system prepared from hog liver (98). Subsequently, a number of investigators have shown that 24(S),25-expoylanosterol, 24(S),25-epoxycholesterol, and 25-hydroxycholesterol can be derived via the cyclization of DOS in mammalian systems (35,38). 25-Hydroxycholesterol and 24(S), 25-epoxycholesterol have been found in cultured fibroblasts (39) and the latter oxysterol was isolated from human liver (38) giving support to the suggestion that it may participate in the regulation of hepatic cholesterol metabolism in vivo.

A schematic comparison of the dioxidosqualene pathways found in plants and animals is presented in Figure 2. As early as 1970 DOS was shown to be cyclized by microsomes from bramble tissues grown in vitro, to form 24,25-epoxycycloartenol (98,99). In Ononis spinosa, DOS is the precursor of α-onocerin under anaerobic conditions (100). In a related study, the investigation of the squalene-2,3;22,23-diepoxide-α-onocerin cyclase enzyme system from Ononis spinosa root has provided evidence for the formation of the intermediate pre-onocerin, which contains a C-24,25 epoxide function originating from DOS (101). Agaiol, the major steroidal triterpenoid of Aglaia ordorata has the 24(S) configuration at the C-24,25 epoxide and may have been formed from DOS with the (22S) configuration (102). 25(27)-Dehydrolanost-8-en-3ß-ol, isolated from Cerus

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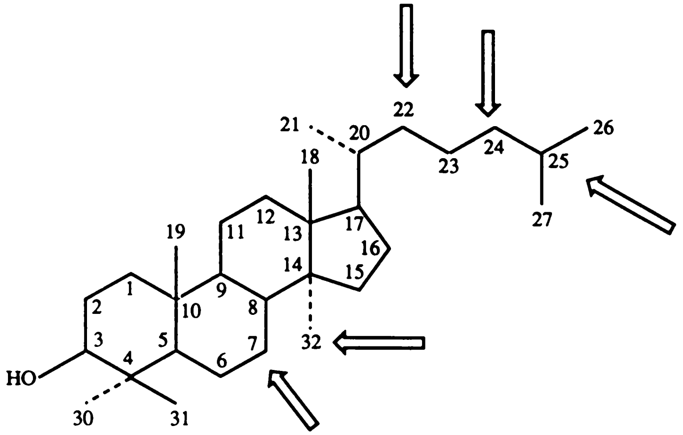
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Figure 1. Designations of carbon atoms in the sterol nucleus and side chain and indicated sites of primary metabolism under discussion.

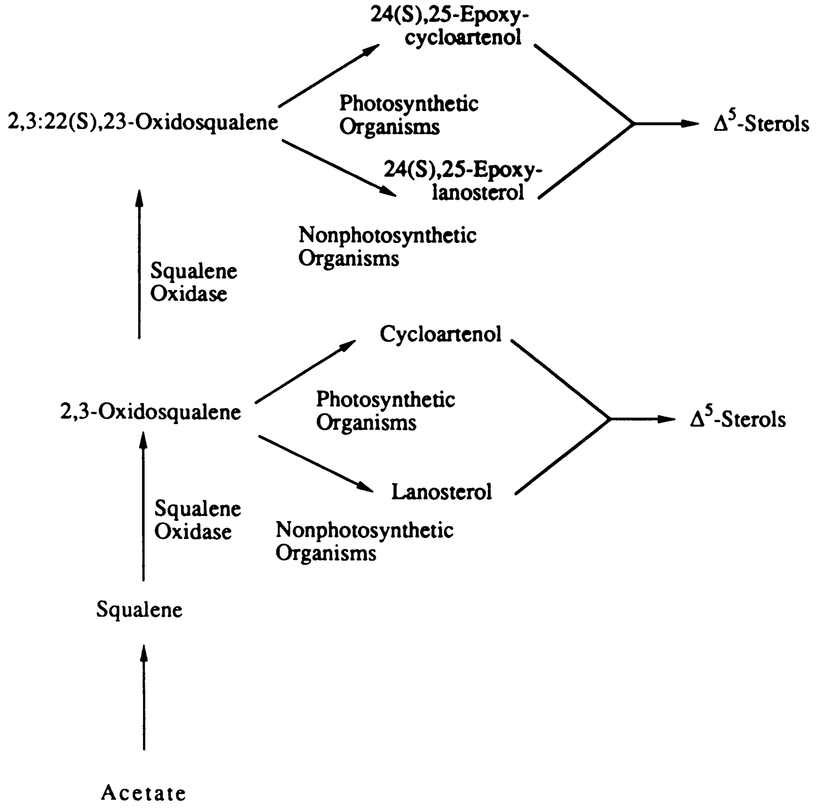
24(S),25-Epoxy-cycloartenol
Photosynthetic Organisms
2,3:22(S),23-Oxidosqualene
24(S),25-Epoxy-lanosterol
Δ^5-Sterols
Nonphotosynthetic Organisms
Squalene Oxidase
Cycloartenol
Photosynthetic Organisms
2,3-Oxidosqualene
Δ^5-Sterols
Lanosterol
Squalene Oxidase
Nonphotosynthetic Organisms
Squalene
Acetate

Figure 2. The biosynthesis of sterols and oxysterols in plants, animals and microorganisms.

giganteus, was proposed to arise from the DOS pathway via opening of the C-24,25 epoxide to give the C-25 hydroxy derivative which is then dehydrated to form the $\Delta^{25(27)}$ double bond (103). DOS has been found in rat liver homogenates (104) and isolated from yeast in 1977 (105) together with the cyclized product 24,25-epoxylanosterol (105. In a related study (Figure 3), it was shown that 2(R,S),3-epiminosqualene was oxidized and cyclized to 24(R,S),25-epiminolanosterol in Gibberella Fujikuroi (106) and, furthermore it was subsequently found that the 24(R,S),25-epiminolanostrol was metabolized via opening of the aziridine ring to 25-aminolanosterol in the same organism (107). This latter study demonstrates that the enzymes involved in the DOS pathway are non-specific. The literature contains many examples of steroids and steroidal triterpenoids, isolated from plants and microorganisms, which contain an oxygen function on C-24 and/or C-25 (59, 108-121). The presence of these compounds in such numbers, indicates the possible widespread occurrence and use of the DOS pathway in these organisms.

The side chain hydroxylation of cholesterol at C-24, C-25 and at C-26 is known to occur in rat liver mitochondria in vitro (122,123). 26-Hydroxylation is believed to constitute the initial step in a minor pathway in bile acid formation (124) whereas the importance of 24- and 25-hydroxylation of cholesterol is unknown (125). Steroids related to cholesterol such as sitosterol and 5ß-cholestan-3ß-ol have been reported to be bile acid precursors (126,127). Purified cytochrome P-450, prepared from rat liver microsomes, was found to catalyze efficient 25-hydroxylation of 5ß-cholestan-3α ,7α ,12α-triol as well as vitamin D_3 (128,129). A number of steroids and steroidal triterpenoids have been isolated from plants and microorganisms which contain a single oxygenated functional group at C-25 (121,130-132).

As described above, plants, animals and microorganisms possess the biosynthetic ability to produce diverse types of oxysterols in abundance. The presence of these substances might suggest a regulatory role in the life cycle of these organisms. A recent study found that 24,25(R,S) epoxylanosterol has an inhibitory effect on sterol biosynthesis in Saccharomyces cerevisiae (strain G204, HEM1 HIS3) (133). These preliminary results indicate the potential for future studies in this area, especially in light of the regulatory role oxysterols have demonstrated in mammalian systems.

Conclusions

A great deal of effort has been spent on the study of biosynthesis of oxysterols and their regulatory role in mammalian biochemistry. The extensive study of the biosynthesis of oxysterols and their regulatory role in

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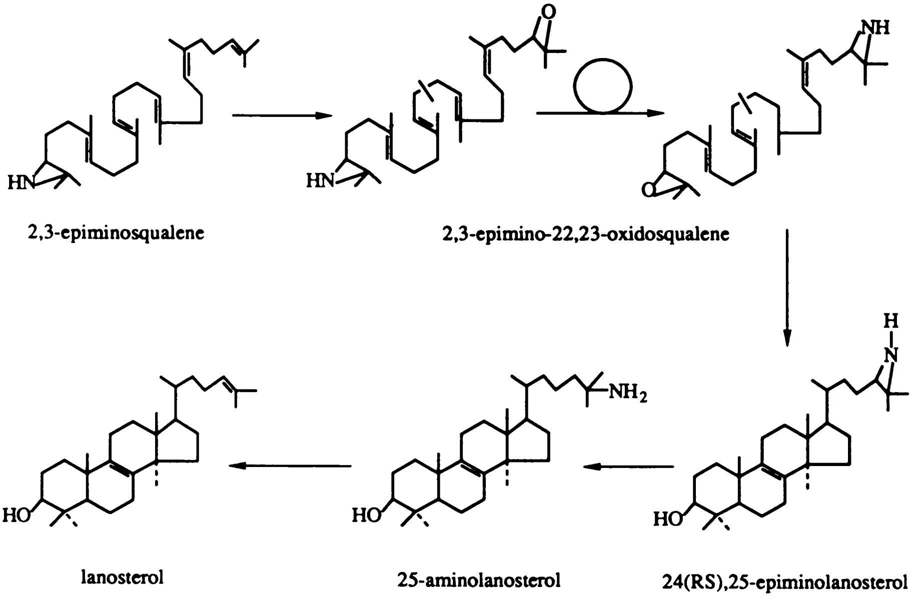
HN
2,3-epiminosqualene
O
HN
2,3-epimino-22,23-oxidosqualene
NH
O
H
N
NH₂
HO
HO
HO
lanosterol
25-aminolanosterol
24(RS),25-epiminolanosterol

Figure 3. The metabolism of $2(R,S),3$-epiminosqualene in Gibberella fujikuroi.

derivatives, in preventing heart disease has been an underlying goal of these studies. As this report has demonstrated, the origins of these biosynthetic pathways can be found in lower organisms and in some instances have been known before their discovery in animal systems. Future research will certainly reveal many new discoveries in the area of oxysterol biosynthesis and function.

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Chapter 12

Enzyme Systems

Use in the Development of Sterol Biosynthesis Inhibitors as Agrochemicals

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Although the sterol biosynthesis inhibitors currently in use as agrochemicals were discovered by classical screening procedures, it is now clear that the development of new ones can be facilitated by use of procedures which allow easy comparison of the efficacy of candidate compounds as inhibitors of the particular target enzyme. This paper describes assay procedures, based upon enzyme preparations derived from a high sterol strain of *Saccharomyces cerevisiae*, for comparing the efficacy of compounds as inhibitors of squalene epoxidase, 2,3-epoxysqualene:lanosterol cyclase, sterol 14-demethylase, sterol 4-demethylase, sterol Δ^{14}-reductase and sterol $\Delta^{8}\rightarrow\Delta^{7}$-isomerase.

Over the past quarter-century compounds that inhibit sterol biosynthesis have found considerable use as agrochemicals. These compounds are usually called sterol-biosynthesis-inhibitors or SBIs. Their most important use has been as fungicides, protecting crop plants from such phytopathogens as powdery mildews and rusts (*1*). In this context their selective toxicity towards fungi appears to be due, in some cases, to the the greater sensitivity of the fungal enzyme targeted (*2*) and, in others, to the greater tolerance of the host plant membranes to the abnormal sterols that are incorporated into them (*3*). Another use of some of these compounds has been as plant growth regulators. In particular triazoles, such as paclobutrazol, uniconazol and triapenthenol, which are inhibitors of the sterol 14-demethylase and, as a

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consequence, have fungicidal properties, inhibit the vegetative growth of a wide range of plants without affecting their generative growth (*4*). They do this by inhibiting the *ent*-kaurene to *ent*-kaurenoic acid steps of gibberellin biosynthesis (*5-8*) which are catalysed by cytochrome P-450 enzymes (*9,10*) that are rather similar the sterol 14-demethylase.

Virtually all of these compounds were discovered by classical greenhouse screening procedures, whose great strength is their ability to give a collective measure of several different parameters, such as uptake, translocation and inhibition of the target enzyme, at once. Although such a measurement of the overall efficacy of the compound must be made at some stage in its development as an agrochemical, it is now recognised that the development of better compounds within existing SBI classes and the discovery of new SBI classes can be facilitated by the use of assay procedures which measure solely their efficacy as inhibitors of the target enzyme. This has become particularly important with the move, in recent years, towards the rational design of inhibitors of particular steps in sterol biosynthesis (*11-15*).

This paper describes the development of assay procedures that can be used to compare the efficacy of compounds as inhibitors of enzymes catalysing particular steps in the post-squalene segment of the fungal sterol biosynthetic pathway. Since these procedures are intended for the routine screening of numerous compounds, emphasis has been placed on their ease of use. Thus the enzyme systems are easy to prepare, the substrates are either commercially-available or relatively easy to prepare and the measurement of the particular reaction(s) under investigation is straightforward.

Preparation of the Enzyme Systems

The enzyme systems were prepared from a high-sterol strain of yeast (*Saccharomyces cerevisiae*, N.C.Y.C. 739), a 10% inoculum of which was grown statically, and therefore virtually anaerobically, for 48 hr at 30°C in a 2 litre conical flask filled to the neck with a medium containing per litre: $(NH_4)_2SO_4$, 2g; KH_2PO_4, 2g; Na_2HPO_4, 0.5g; $MgSO_4.7H_2O$, 0.25g; $MnSO_4.4H_2O$, 0.025g; D-glucose, 20g and yeast extract (Difco), 1g. The cells were harvested by centrifugation at 1000*g*, washed twice with 0.1M phosphate buffer, pH 6.2 and then resuspended in the same buffer to which 10%(w/v) D-glucose had been added, and either shaken at 160 rpm in a gyrotary shaker at 25°C or vigorously aerated with filtered, moistened air at 28-30°C in the presence of BDH silicone antifoaming agent (1ml/l), for 2.5 hr. The resulting aerobically-adapted cells were harvested by centrifugation at 1000*g* and washed twice with 0.1M phosphate buffer, pH 6.8. At this point the cells could be either homogenised at once or stored overnight at -20°C if necessary. The cells, after slowly thawing to room temperature if they had been stored, were resuspended in a homogenisation medium consisting of 0.1M phosphate buffer, pH 6.8, containing 30mM nicotinamide, 5mM $MgCl_2$ and 5mM *N*-acetyl-L-cysteine and then homogenised. This was accomplished either by passing the cell suspension (0.75ml medium/g wet wt. cells) through a precooled French pressure cell operating at 20000

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psi (0.1379 GPa) or by subjecting the cell suspension (2ml medium/ g wet wt. cells) to disruption in a Bronwill MSK cell mill cooled by liquid CO_2. The resulting homogenate was centrifuged at 1000*g* for 10 min. at 4^oC and the supernatant so obtained recentrifuged at 8000*g* for 20 min. at 4^oC. The 8000*g* supernatant was filtered through glass wool to remove any floating lipid layer and was used immediately as the S_8 enzyme system. However for the sterol $\Delta^8 \rightarrow \Delta^7$-isomerase assay the homogenate was centrifuged at 2500*g* for 10 min. at 4^oC and the resulting supernatant converted into an acetone powder by the method described by Moore and Gaylor (*16*). In the assay the acetone powder was used as a 10mg/ml suspension in 0.1M phosphate buffer, pH 6.8, containing 5mM *N*-acetyl-L-cysteine. The S_8 enzyme system is capable of catalysing the conversion of 3*R*-mevalonic acid (MVA) into ergosterol (*17*) whilst the acetone powder suspension contains an active sterol $\Delta^8 \rightarrow \Delta^7$-isomerase.

Assay of Inhibitors of Squalene Oxidase and 2,3-Epoxysqualene:Lanosterol Cyclase

This assay depends on (i) the ability of the S_8 enzyme system to convert 3R-mevalonic acid (MVA) into ergosterol and (ii) the easy separation of squalene and 2.3-epoxysqualene from each other and from sterols by thin layer chromatography (t.l.c.).

A series of incubation mixtures were set up in ground glass-stoppered test-tubes. Each contained the following components in a final volume of 1ml: 0.9ml S_8 enzyme system; 0.5µCi 3*RS*-[2-^{14}C]MVA.DBED salt; 3µmol ATP; 1µmol FAD; 1µmol NADPH; 3µmol NAD^+; 2µmol L-methionine; 3µmol reduced glutathione; 5µmol $MgCl_2$; 1µmol $MnCl_2$; 5µmol D-glucose-6-phosphate (G-6-P); 2.3nkat G-6-P dehydrogenase and 5µl of methanol (control incubations) or 5µl of a methanolic solution containing the amount of candidate epoxidase or cyclase inhibitor necessary to give the required concentration in the final 1ml incubation mixture (test incubations). Each incubation mixture was agitated for 15 sec., using a whirlimix, immediately after the addition of the final ingredient, the MVA, so as to mix the constituents thoroughly and to saturate it with oxygen. The incubation mixtures were then incubated for 1 hr. at 30^oC with constant, vigorous shaking to ensure continued oxygenation. The mixtures were then saponified by heating at about 80^cC for 20 min. in the presence of 2ml of ethanol and one pellet of KOH (giving a KOH concentration of approx. 1M in the saponification mixture). The saponification mixtures were then cooled and diluted by the addition of 4ml of water; then each was extracted three times with 3ml of light petroleum, b.p. $40\text{-}60^oC$, and the extracts combined and evaporated to dryness under nitrogen.

The resulting unsaponifiable lipids (which include squalene, 2,3-epoxysqualene and sterols) from each incubation mixture were then subjected to t.l.c. on silica gel $60F_{254}$ plastic-backed plates using ethyl acetate/cyclohexane (1/4, v/v) for development. The t.l.c. plates were radioautographed for 3-4 days to locate the radioactive zones corresponding to squalene, 2,3-epoxysqualene and the sterols. These zones were then cut out and radioassayed by liquid scintillation counting.

The percentages of the total radioactivity of the unsaponifiable lipids that were present in the squalene (designated %Sq) and 2,3-epoxysqualene (designated %SqO) of each incubation mixture were then determined and the dose-reponse curves obtained by plotting %Sq and %SqO against inhibitor concentration. An inhibitor of squalene epoxidase causes an increase in the %Sq as its concentration increases; similarly an inhibitor of 2,3-epoxysqualene:lanosterol cyclase causes an increase in %SqO. By judicious choice of the concentration range assayed, the I_{50} value of the inhibitor (i.e., the concentration causing 50% inhibition) can be determined.

Essentially similar procedures to that described above for the the assay of squalene epoxidase have been developed using *S.cerevisiae* microsomal fraction as the source of enzyme and [^{14}C]farnesyl pyrophosphate as the substrate (*18*) and using particulate preparations from *Candida albicans* and *C. parapsilosis* and [^{3}H]squalene (*19,20*).

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Assay for Inhibitors of Sterol 14-Demethylase and Sterol 4-Demethylase

The assay procedure described above, using the S_8 enzyme system and 3*RS*-[2-^{14}C]MVA.DBED salt as the substrate, has been used for the assay of 14-demethylation-inhibiting fungicides and candidate DMI fungicides (*2*). This is possible because: (i) the S_8 enzyme system can catalyse the conversion of 3*R*-mevalonic acid into ergosterol, (ii) the normal sequence of sterol demethylation in yeast is 4,4,14-trimethyl sterol → 4,4-dimethyl sterol → 4α-methyl sterol → 4-demethyl sterol, with DMI fungicides blocking the first of these steps, (iii) the S_8 enzyme system is apparently unable to catalyse out-of-normal-sequence demethylations during the incubation period used and (iv) the t.l.c. system used is able to separate sterols into three classes on the basis of the number of methyl groups attached to C-4, namely the 4,4-dimethyl sterols (which include 4,4,14-trimethyl sterols), 4-monomethyl sterols (i.e., 4α-methyl sterols) and 4-demethyl sterols.

From this, it is apparent that when a DMI fungicide is present in the [2-^{14}C]MVA/S_8 incubation mixture and the above assay procedure followed, there will be an increase of radioactivity in the 4,4-dimethyl sterol t.l.c. zone (due to 4,4,14-trimethyl sterol) and a decrease of radioactivity in the 4-demethyl sterol t.l.c. zone, relative to a no-DMI control. Moreover the greater the degree of inhibition the more accentuated these differences will become. This is demonstrated in Figure 1 which is a photograph of the radioautograms taken after the t.l.c. separation of the lipid extracted from control (Con) and test incubations (0.5 -100μM inhibitor concentrations) during the assay of Etaconazole, a DMI fungicide (Di, 4α and De = 4,4-dimethyl-, 4α-methyl- and 4-demethyl sterols respectively).

A quantitative measure of the change in distribution of radioactivity between the 4,4-dimethyl and 4-demethyl sterol fractions caused by inhibition of 14-

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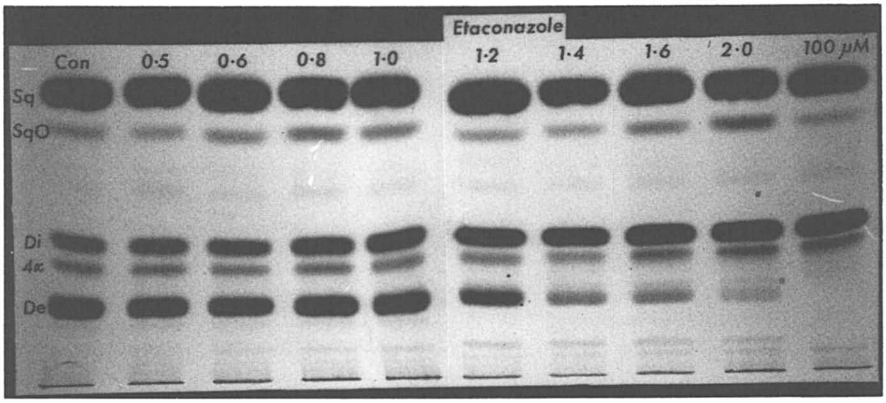

Figure 1. Assay of Etaconazole as an inhibitor of the sterol 14-demethylase, showing the radioautogram of the t.l.c. of the unsaponifiable lipids extracted from control (Con) and inhibited (0.5 - 10CμM Etaconazole) incubations. (Sq = squalene; Di = 4,4-dimethyl sterols; 4α-methyl sterols; De = 4-demethyl sterols)

demethylation can be obtained by radioassay of the t.l.c. zones by liquid scintillation counting and determination of the percentage of the radioactivity in the total sterols that is present in the 4,4-dimethyl sterols (designated %Di). A dose response-curve can then be obtained by plotting %Di against the DMI concentration. From this an I_{50} value can be determined by making use of the fact that the control and maximum %Di values represent 0 and 100% inhibition respectively; in this context the maximum %Di value is that which is obtained when the %Di values become constant at high DMI concentrations.

A similar procedure to this has been developed using a 1500*g* supernatant derived from a *C. albicans* homogenate as the enzyme system and 3*RS*-[2-^{14}C]MVA.DBED salt as the substrate (*21*), and has been used for the assay of *N*-substituted imidazole antifungals.

This assay procedure can only be used when it is certain that the compound under test is an inhibitor of the sterol 14-demethylase, as was the case with the compounds tested in refs. (*2*) and (*21*) and with many *N*-substituted imidazole and triazole fungicides and candidate antifungals. This follows because a compound that inhibits the sterol 4-demethylase will produce precisely the same effect in this assay procedure as a 14-demethylase inhibitor. This means that if a search for fungicides, or other useful agrochemicals, that exert their primary biological activity by inhibiting sterol 4-demethylation is to be assisted by an enzyme assay procedure, that procedure will have to be more specific than the one just described. A procedure of this kind could be based upon the measurement of the reduction in $^{14}CO_2$ evolution from a sterol substrate that is labelled with ^{14}C in both C-4 methyl groups and devoid of a C-14 methyl group, when it is incubated with an enzyme preparation, like the S_8 enzyme system, which contains both sterol 4- and 14-demethylases

Such a substrate could be [30,31-$^{14}C_2$]4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol which can be synthesised from commercially-available 7-dehydrocholesterol and $^{14}CH_3I$ as starting materials. This synthesis involves the Oppenauer oxidation (*22,23*) of 7-dehydrocholesterol, followed by reaction of the resulting cholesta-4,7-dien-3-one with $^{14}CH_3I$ in the presence of *tert*-butoxide (*24*) to give [30,31-$^{14}C_2$]4,4-dimethylcholest-5,7-dien-3-one. Reduction of the latter with $LiAlH_4$ (*25*) to the corresponding 3β-hydroxy sterol, followed by acetylation and HCl isomerisation (*25*) yields the acetate of [30,31-$^{14}C_2$]4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol, from which the free sterol can be obtained by saponification.

The use of this compound with the S_8 enzyme system would require the presence of NADPH, an NADPH-generating system and NAD^+ as cofactors. The incubation mixture would require to be shaken to ensure adequate oxygenation. Each incubation would have to be carried out in a closed system, with provision for sweeping the $^{14}CO_2$, released during the 4-demethylation reaction, through a combined CO_2-trapping/liquid scintillator solution, such as Oxosol-^{14}C (National Diagnostics, N.J.), after the incubation period was over.

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Assay for Inhibitors of the Sterol Δ^{14}-reductase

This assay depends upon: (i) the ability of the sterol Δ^{14}-reductase present in the S_8 enzyme system to catalyse the reduction of the Δ^{14}-double bond of 24-methylene ignosterol (M-igno; 5α-ergosta-8,14,24(28)-trien-3β-ol), despite the fact that 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol is its natural substrate, and (ii) the fact that M-igno absorbs light maximally at 250nm (see Figure 2a) due to the presence of the $\Delta^{8,14}$-heteroannular conjugated diene. The assay measures the disappearance of M-igno, as its Δ^{14}-double bond is reduced, by determining the diminution in absorbance at 250nm that has occurred after a 2 hr incubation with the S_8 enzyme system.

M-igno was prepared biosynthetically by aerobically-adapting statically-grown (i.e., virtually anaerobically-grown) *S. cerevisiae* (N.C.Y.C. 739) for 4-6 hr at 30°C in 0.1M phosphate buffer, pH 6.8, containing 10% D-glucose and 200μM Fenpropidin. Fenpropidin is a fungicide that inhibits the sterol Δ^{14}-reductase far more efficiently than the sterol $\Delta^{8}\rightarrow\Delta^{7}$-isomerase (*26,27*) and causes the accumulation of M-igno in the yeast as the squalene, which had built up during the period of anaerobic growth, is converted into sterol during aerobic adaptation. The cells were then harvested, saponified and the unsaponifiable lipid extracted. The latter was then chromatographed on acid-washed, Brockmann Grade 3 alumina using successive volumes of light petroleum, b.p. 40-60°C (P), 4% peroxide-free diethyl ether (E) in light petroleum (4% E/P), 8% E/P, 12% E/P, 16% E/P, 20% E/P and 100% E for development. The 16% E/P fraction gave M-igno that was more than 90% pure (by spectrophotometry) and was used as the substrate for the assay. Its only u.v.-absorbing contaminant was ergosterol.

A series of incubation mixtures was set up in ground glass-stoppered test-tubes. Each tube contained the following components in a final volume of 1ml: 0.9ml S_8 enzyme system; 350nmol M-igno in 50μl ethanol; 1μmol NADPH; 5μmol G-6-P; 2.3nkat G-6-P dehydrogenase and 10μl ethanol (control incubations) or 10μl of an ethanolic solution containing the amount of candidate 14-reductase inhibitor necessary to give the required concentration in the final 1ml incubation mixture (test incubations). Two types of control incubations were used, namely (i) a zero-time control, to which 2ml of ethanol were added immediately to denature the S_8 enzymes and thereby prevent any reduction of the M-igno; this gave a measure of the quantity of M-igno present at the start of the incubation and ought to be equal to the quantity of M-igno added, and (ii) a control that was incubated for the full incubation time; this gave a measure of the quantity of M-igno that was reduced in the absence of any Δ^{14}-reductase inhibitor, i.e., the maximum conversion.

After thorough mixing, using a whirlimix, the incubation mixtures were incubated for 2 hr at 30°C in the dark with continuous shaking. Then 2ml of ethanol and 2 pellets of KOH were added to each tube and, after shaking to dissolve the KOH (giving a KOH concentration of approx. 2M in the saponification mixture), they were

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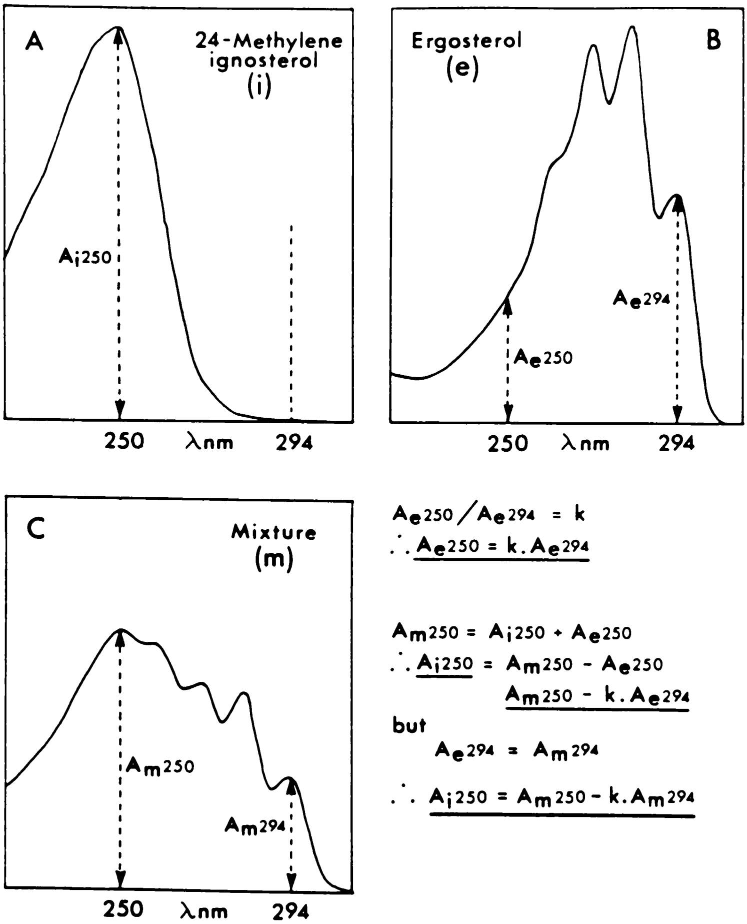

$$A_e250/A_e294 = k$$

$$\therefore \underline{A_e250 = k.A_e294}$$

$$A_m250 = A_i250 + A_e250$$

$$\therefore \underline{A_i250} = A_m250 - A_e250$$

$$\underline{A_m250 - k.A_e294}$$

but

$$A_e294 = A_m294$$

$$\therefore \underline{A_i250 = A_m250 - k.A_m294}$$

Figure 2. Absorption spectra of 24-methylene ignosterol (A), ergosterol (B) and a mixture of the two (C) in ethanol. (A_i250, A_i294, A_e250, A_e294, A_m250, and A_m294 = Absorbance of 24-methylene ignosterol, ergosterol and a mixture of the two sterols respectively at 250 or 294nm)

allowed to stand overnight in the dark at room temperature to effect a cold saponification. The saponification mixtures were each diluted by the addition of 4ml water, extracted twice with 3ml of light petroleum, b.p. 40-60^{c}C, the extracts combined and passed down a 1g column of acid-washed, Brockmann Grade 3 alumina. The sterols, mainly residual M-igno and ergosterol, were eluted from the alumina with 10ml of peroxide-free diethyl ether. The latter was evaporated off under N_2 and the residue dissolved in 5ml of ethanol. The absorbance of this solution was then measured at 250 and 294nm.

The assay procedure takes advantage of the presence of ergosterol in the substrate sterol and the S_8 enzyme system by using it as an internal standard to correct for any difference in the completeness of sterol extraction amongst the incubation mixtures. This is possible because: (i) ergosterol has a characteristic u.v. absorption spectrum (see Figure 2b) with peaks at 272, 282 and 294nm and a shoulder at 262nm, (ii) M-igno has no significant absorption at 294nm, which means that in a mixture of M-igno and ergosterol (see Figure 2c) the absorption at 294nm is due solely to ergosterol, thus giving a measure of the ergosterol present, and (iii) though ergosterol absorbs appreciably at 250nm, where M-igno has its absorption maximum, the absorption at 250nm of a mixture of the two sterols that is solely due to M-igno can easily be calculated because the ratio of the absorbances of 250 and 294nm is a constant (see Figure 2). Thus, by determining the absorbances at 250 and 294nm of the sterol extracted from the zero-time control incubation and knowing the quantity of M-igno that has been added to it (which is the same for all the incubation mixtures), it is possible to calculate the total quantity of ergosterol that is present in it, and all the incubation mixtures, at the start of the incubation period. Because this quantity does not change during the course of the incubation, the absorbance at 294nm of the sterol extracted from the incubation mixture after the incubation period is complete can be used to calculate the fractional recovery of ergosterol. On the assumption that the fractional recovery of all the sterols from the incubation mixture are the same and being able to calculate what part of the measured absorbance at 250nm is due to M-igno, it is possible to determine the total quantity of M-igno that was present in the incubation mixture at the end of the incubation period. In these calculations, which were computerised, the molar absorption coefficient of M-igno was taken to be 18000 on the basis of values determined for very similar $\Delta^{8,14}$-sterols (*28-30*).

Thus, knowing the amount of M-igno present in each incubation mixture at the start of the incubation period and having calculated the amount that remains at the end, the percentage of M-igno that has been converted into its reduction product can be calculated. Then, knowing the percentage conversion for each inhibitor-containing incubation mixture and that for the no-inhibitor control, the percentage inhibition of the sterol Δ^{14}-reductase caused by each inhibitor concentration can be calculated. From a plot of percentage inhibition versus inhibitor concentration the I_{50} of the inhibitor can be determined.

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Assay for Inhibitors of the Sterol $\Delta^8 \rightarrow \Delta^7$-isomerase

This assay depends upon: (i) the relatively easy preparation of fecosterol (5α-ergosta-8,24(28)-3β-ol), the natural substrate of the sterol $\Delta^8 \rightarrow \Delta^7$-isomerase in all fungi, (ii) the ability of the yeast acetone powder preparation to catalyse the conversion of fecosterol into episterol (5α-ergosta-7,24(28)-dien-3β-ol), and (iii) the separability of these two sterols by capillary gas-liquid chromatography (g.l.c.) on methyl silicone liquid phases. It is also facilitated by the virtual absence from the acetone powder of endogenous ergosterol, which would otherwise have tended to swamp the g.l.c. separation of fecosterol and episterol.

Fecosterol was prepared biosynthetically by aerobically-adapting statically-grown (i.e., virtually anaerobically-grown) *S. cerevisiae* (N.C.Y.C. 739) for 18 hr at 30°C in 0.1M phosphate buffer, pH 6.8, containing 10% D-glucose and 200μM Tridemorph. Tridemorph is a fungicide that inhibits the sterol $\Delta^8 \rightarrow \Delta^7$-isomerase much more efficiently than the sterol Δ^{14}-reductase (*26,27*) and consequently causes the accumulation of fecosterol in the yeast as the squalene, which had built-up during the period of anaerobic growth, is converted into sterol during aerobic adaptation. The cells were then harvested, saponified and the unsaponifiable lipid extracted. The 4-demethyl sterols were isolated by the t.l.c. system described earlier and then acetylated. The sterol acetates were then subjected to argentation t.l.c. using 6% $AgNO_3$-impregnated silica gel plates which were developed twice with toluene. The major zone (R_f 0.65), corresponding to fecosterol acetate, was extracted and converted back to the free sterol by saponification. It was confirmed as fecosterol by mass spectrometry and shown to be 97% pure by g.l.c. (*27*).

A series of incubation mixtures was set up in ground glass-stoppered test-tubes. Each tube contained the following components in a final volume of 2ml: 1.985ml acetone powder suspension; 20nmol fecosterol in 5μl ethanol; and 10μl of ethanol (control incubations) or 10μl of an ethanolic solution containing the amount of candidate sterol $\Delta^8 \rightarrow \Delta^7$-isomerase inhibitor necessary to give the required concentration in the final 2ml incubation mixture (test incubations). After thorough mixing using a whirlimix, the incubation mixtures were incubated for 3 hr at 30°C with continuous shaking.

The reaction in each tube was terminated by the addition of 4ml of ethanol and the sterols then extracted three times with 3ml *n*-hexane. The combined *n*-hexane extracts were evaporated to dryness under N_2. The resulting residue was dissolved in 1ml of toluene and 0.5μl aliquots subjected to g.l.c. on OV-1 or SGE BP-5 WCOT quartz columns (15m x 0.3mm i.d.) using helium flowing at 8ml/min as the carrier gas and a column oven temperature programmed from 100°C (1 min) to 260°C (1 min) to 270°C (5 min) at 40°C/min and 2°C/min respectively. The output of the flame ionisation detector, operating at 300°C, was fed into a computing integrator. The percentage fecosterol → episterol conversion was calculated as follows: area counts in the episterol peak x 100 / area counts in the sum of the episterol and fecosterol peaks. On the assumption that the percentage conversion value in the no-inhibitor control incubation represents 100% conversion in the particular experimental run, the percentage inhibition values were calculated as follows: [% conversion in the control incubation - % conversion in the test incubation] x 100 / % conversion in the control incubation. The percentage inhibition values so obtained were then plotted against fungicide concentration and from the resulting curve the I_{50} value determined.

Interpretation and Use of I_{50} Values obtained in these Assays

Because of variation from enzyme preparation to enzyme preparation the I_{5C} value determined for a given inhibitor in any of the assays described has no meaning in absolute terms. It is only meaningful in relative terms, i.e., when it is compared with the I_{50} values of other inhibitors measured at the same time using the same preparation of the enzyme system. At first sight this seems to be a severe limitation of the usefulness of the assays because laboratory logistics limit the number of candidate inhibitors that can be tested in a single experiment. However comparability from experiment to experiment can be obtained by selecting one inhibitor to act as a standard for a particular assay. All candidate inhibitors are compared with this standard by including the standard inhibitor in every experiment and the I_{50} measured for it adjusted to some arbitrary, but realistic, constant value by multiplying it by the factor necessary to do this. This factor will vary from experiment to experiment as the activity of the enzyme preparation varies. The I_{50} values measured for all the candidate compounds are adjusted proportionately by multiplying them by the factor appropriate to the experiment in which they were assayed. In this way it is possible to build up a "league table" in which the standard and candidate inhibitors are placed in ranking order.

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Chapter 13

2,3-Oxidosqualene Cyclase and Squalene Epoxidase as Target Enzymes for the Development of New Sterol Biosynthesis Inhibitors

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10125 Torino, Italy**

2,3-Oxidosqualene cyclase and squalene epoxidase are two key enzymes involved in the biosynthesis of sterols in animals, plants and yeasts. These enzymes represent a good target for developing selective fungicide, antimycotic and hypocholesterolemic drugs. Various classes of inhibitors of 2,3-oxidosqualene cyclase, many of which can be considered analogues of high energy intermediates, and of squalene epoxidase are examined and the possible mechanisms of inhibition are discussed.

There has recently been considerable interest in the discovery and development of new drugs which interfere with the biosynthesis of sterols (1-4). In particular, a wide variety of fungicides and antimycotics of different chemical structure share in common the ability to inhibit ergosterol biosynthesis. Sterols from higher plants could also be useful targets for developing growth regulators and herbicides based on similar modes of action (5-7). The more recently discovered sterol biosynthesis inhibitors designed on a rational basis could represent a very useful lead for developing selective toxicants in agriculture.

We and others have attempted to design new sterol inhibitors which closely resemble the postulated carbocationic high-energy intermediates (HEI) **3,14-16** of the enzyme-catalysed reactions. We applied this approach to synthesize very potent and selective inhibitors of 2,3-oxidosqualene cyclase, a group of strictly correlated enzymes which convert 2,3-oxidosqualene (SO) **2** to lanosterol **8** in animals and fungi and to cycloartenol **5** in plants (8-14) (Figures 1 and 2). SO cyclase can also cyclize to rigid systems other than sterols as in the production of α-, β-amyrin **13**, parkeol **6** or cucurbitacins (15-17).

We also focused our attention on squalene epoxidase, a non-hemic

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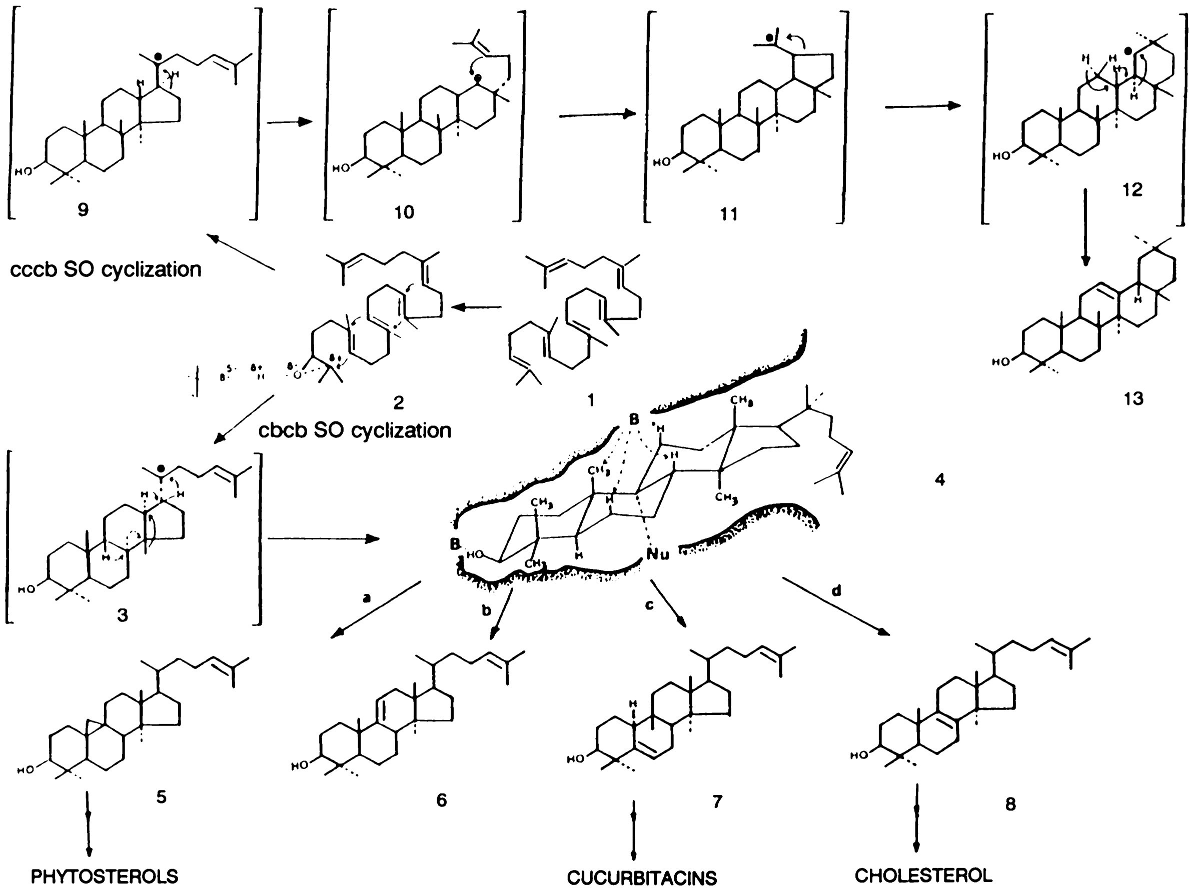
9
10
11
12
13
cccb SO cyclization
2
1
cbcb SO cyclization
3
4
B
Nu
HO
CH_3
H
a
b
c
d
5
6
7
8
PHYTOSTEROLS
CUCURBITACINS
CHOLESTEROL

Fig. 1 Squalene epoxidation and 2,3-oxidosqualene cyclization in animals, higher plants and fungi.

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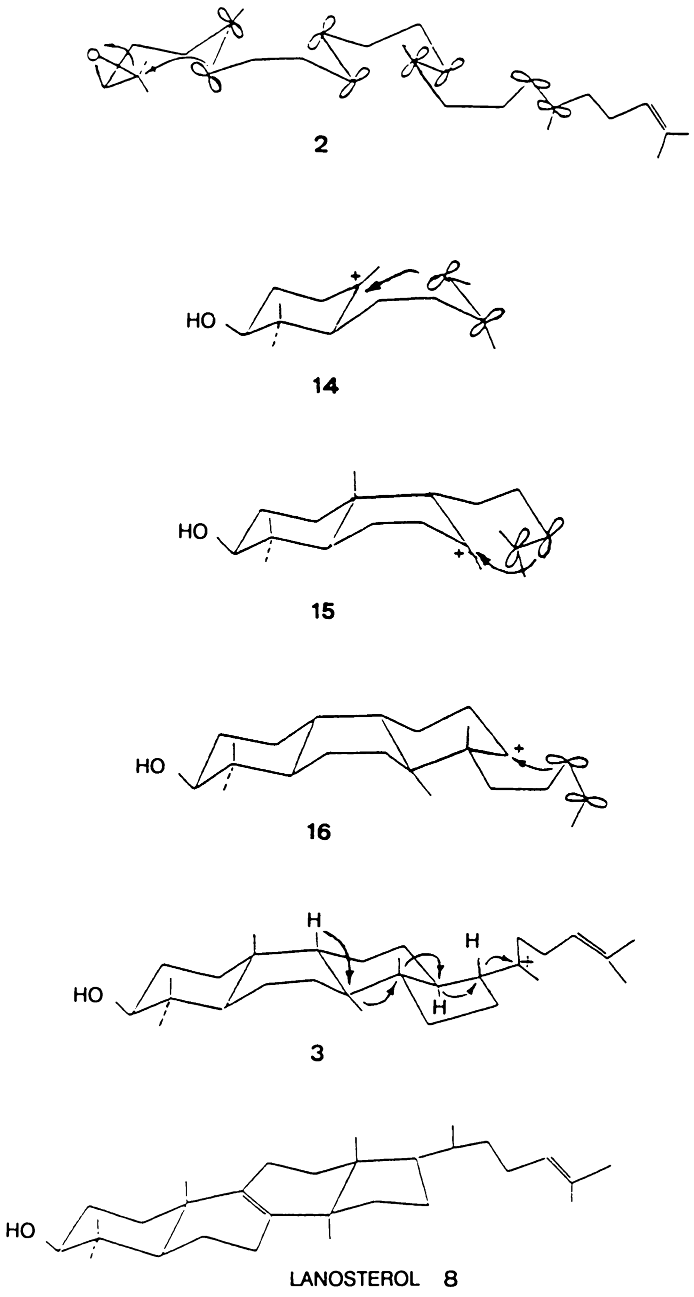

Fig. 2 Proposed mechanism of 2,3-oxidosqualene cyclization.

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flavin-dependent mono-oxygenase which converts squalene **1** to SO **2** in eukaryotes. In this case we designed mechanism-based inhibitors (active site directed) which would be able to be transformed enzymatically into a highly reactive intermediate and, accordingly, to be covalently linked to the active site of the enzyme.

2,3-Oxidosqualene Cyclase Inhibitors

Two fundamental types of cyclase can be envisaged: the cyclase responsible for the chair-boat-chair-boat (cbcb) folded cyclization of SO to lanosterol-type tetracyclic triterpenoids such as lanosterol, cycloartenol, parkeol and cucurbitadienol, and the cyclase which produces pentacyclic triterpenes such α- or β-amyrin, utilizing a cccb folded squalene epoxide (Figure 1).

The group of cyclases involved in the cbcb folded SO cyclization are similar in their mechanism, as probably the same basic group present in the transition state of the enzyme is responsible for the final proton extraction to obtain the desired tetracyclic triterpenes (parkeol **6**, lanosterol **8**, cycloartenol **5**, or cucurbitadienol **7**) (18). The other group of cyclases (such as α- or β-amyrin cyclase) may also be very similar to the lanosterol-type cyclases at least in the part of the enzyme responsible for recognition of the substrate and the following cyclization step. This unifying view suggests that compounds designed as inhibitors of lanosterol cyclase might also inhibit cycloartenol or β-amyrin cyclase.

It is still a matter of controversy whether the cyclization of SO to tetracyclic or pentacyclic triterpenes is a non-stop sequence of events controlled by a single enzyme, as suggested by Eshenmoser (19,20), or whether it requires the formation of many stable intermediates and involves more that one enzyme (21). Van Tamelen, giving a modern unifying view, has proposed that in biological SN_2-like polycyclization of SO, the complete annellation process is not fully concerted but would involve a series of conformationally rigid carbocationic intermediates (22,23). This suggestion may be confirmed by the isolation from Pistacia lentiscus resins of a bicyclic triterpenoid **17** closely related to the C-8 bicyclic carbocation **15** postulated as intermediate during the cyclization of SO to lanosterol (Figure 3) (24).

A theory in line with the formation of stable rigid carbocationic intermediates postulates the existence of regiospecific external point charges in the enzyme promoting the specific closure of the rings during SO cyclization. SO cyclization could thus be neutralized by some anionic-charged residues appropriately located in the active site of the enzyme, rather than forming a chemical linkage with a suitable prosthetic group, as suggested by Cornforth.

Further support for this hypothesis has come from our recent synthesis of squalenoid epoxy vinyl ether **18** bearing an isopentyloxy group in the terminal isoprenic unit of SO (25). Compound **18** was synthesized in order to obtain a derivative that upon incubation with SO cyclase could be cyclized, giving a C-20 ion **19**. This could be neutralized by a suitable nucleophilic group of the enzyme, as postulated by Cornforth, yielding a stable covalent bond **20** (Figure 4). The fact that vinyl ether **18** was a competitive inhibitor of SO cyclase was not in line with this hypothesis.

In conclusion, the overall discussion has reinforced our

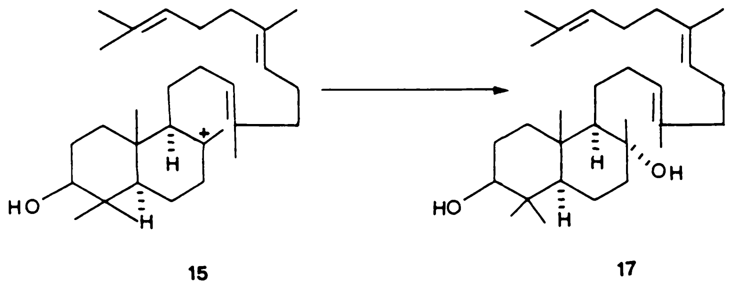
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Fig. 3 Triterpenoid isolated from Pistacia lentiscus related to the C-8 carbocation.

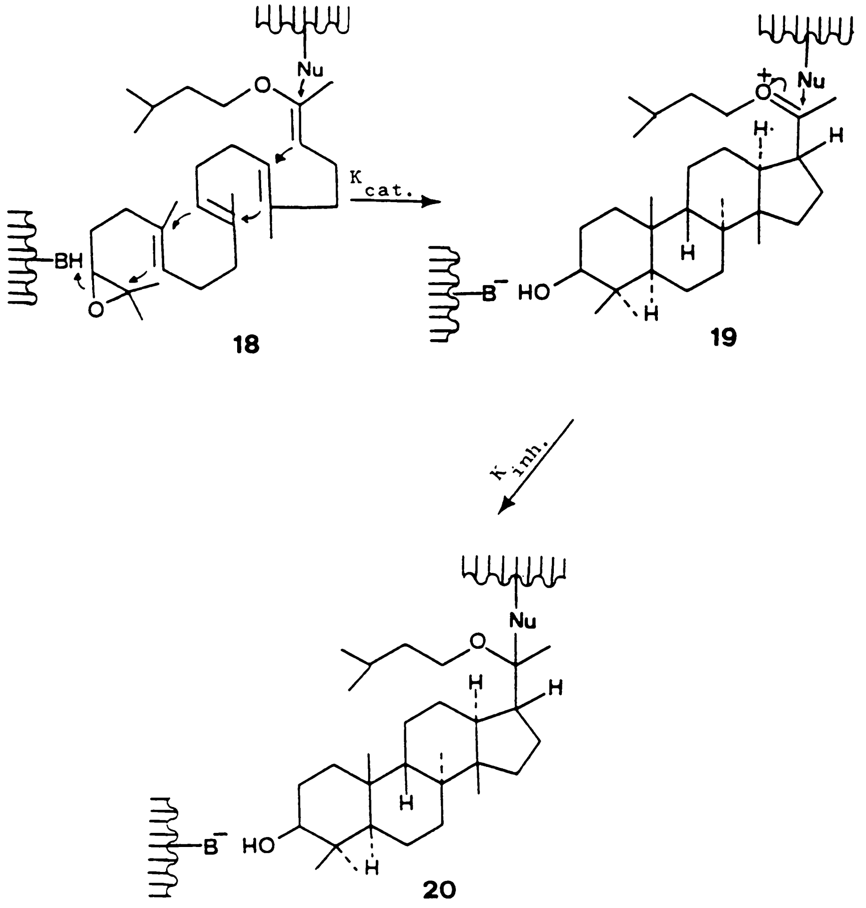
Nu
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Fig. 4 Possible mechanism of action of squalenoid epoxy vinyl ether.

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suggestion that during the conversion of SO to polycyclic triterpenoids, a series of conformationally rigid and stereochemically well-defined carbocationic intermediates are involved. In order to govern the overall cyclization and maintain the substrate in correct stereochemical conformation, the enzyme may assume a suitable conformation to stabilize the carbocationic intermediates through interactions with correctly located ion-pair groups. Another or the same groups of the enzyme could assume the correct position during the following rearrangement of the protosteryl ion, to be able to extract a proton allowing the formation of the final products.

A general strategy applied to prepare potent new inhibitors of SO cyclase has been to mimic the above described carbocations, which can be considered analogues of the enzyme-bound high energy intermediates (HEI) (26-30). Thus, by replacing a positively-charged carbon atom in the structure of the substrate with a nitrogen (protonated at physiological pH), a similar charged species could be obtained.

Initially, we tried to mimic the C-20 carbonium ion of the protosteryl ion by replacing the C-20 atom with a suitable nitrogen atom. The resulting 20-azadammaran-3β-ol or 20-azaprotosterol might behave as a selective inhibitor of SO β-amyrin cyclase or SO cycloartenol cyclase (Figure 5). Unfortunately, the two isomeric 20-azadammaran-3β-ols **21**,**22** behaved as very poor inhibitors of SO β-amyrin cyclase (31).

To explain this apparent contradiction we assumed that, in SO cyclase, the enzyme may adopt very different conformations in order to accomplish the different complex steps responsible both for recognition and cyclization of the substrate and the following rearrangement. Therefore compounds such as the 20-azadammarenols, which could be structurally very far from the conformational ground state of the enzyme able to recognize SO, may not be able to take advantage of the favourable binding interactions occurring in the transition state of the enzyme.

For this reason, we shifted our interest from triterpenes to squalene analogues which could more favourably be recognized by the SO enzyme and mimic the carbocationic transition states. In particular, we have tried to mimic the first carbocationic HEI arising from the opening of the oxirane ring of SO, by designing several series of aza derivatives and testing their *in vitro* effects on SO lanosterol cyclase (rat liver and yeast microsomes), SO cycloartenol cyclase (maize seedling microsomes) and SO β-amirin cyclase (germinating peas) (32-34).

The most interesting properties of azasqualenes may be summarized as follows.

1. The inhibition species is the protonated form of the amine (resulting from the physiological pH *in vivo*) or, more generally, a positively charged species mimicking the first carbocationic intermediate involved in the opening of the oxirane ring of SO. Indeed, derivatives containing quaternary ammonium groups were as strong inhibitors of animal and plant cyclases as the tertiary amines. Compounds such as squalene dimethylamidine **36**, characterized by the delocalization of a positive charge, were excellent inhibitors of pea seedling cyclase, while the 1,1',2-*tris*-*nor*-squalene-3-carboxylic acid **37**, bearing a delocalized negative charge, did not inhibit the cyclase (Figure 6).

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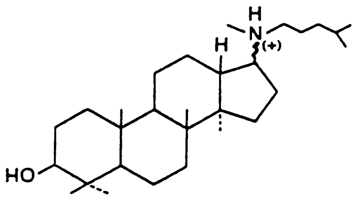
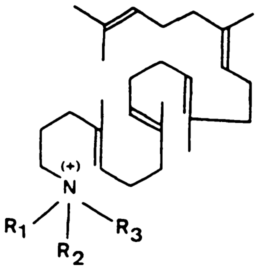
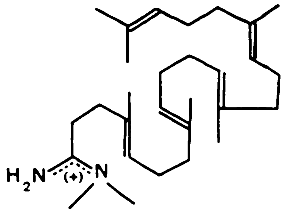


21 a: 17(αH)
22 b: 17(βH)

Fig. 5 Structures of the isomeric 20-azadammaran-3β-ols.





36

23 $R_1 = R_2 = Me; R_3 = H$

24 $R_1 = Me; R_2 = R_3 = H$

25 $R_1 = R_2 = Et; R_3 = H$

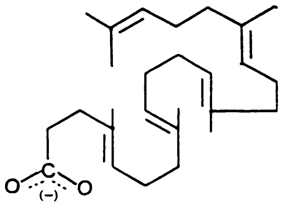
26 $R_1 = Et; R_2 = R_3 = H$

27 $R_1 = R_2 = i\text{-}Pr; R_3 = H$

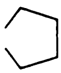
28 $R_1 = i\text{-}Pr; R_2 = R_3 = H$

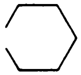
29 $R_1 = R_2 = R_3 = H$

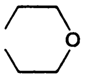
30 $R_1 = R_2 = R_3 = Me$



37

31 $R_1 = H; R_2 = R_3 =$ 

32 $R_1 = H; R_2 = R_3 =$ 

33 $R_1 = H; R_2 = R_3 =$ 

34 $R_1 = R_2 = Me; R_3 = O^-$

35 $R_1 = R_2 = Et; R_3 = O^-$

Fig. 6 Structures of azasqualenes and derivatives.

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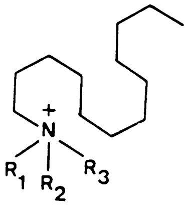
2. The inhibition of SO cyclase is controlled by steric factors at the N-2 centre. In fact, N,N-dimethyl **23** and N,N-diethyl **25** compounds were the most active, the inhibition being particularly strong for the pea enzyme. Inhibition strongly decreased when the N-substituents were bulkier that ethyl **27,28** or the compounds lacked the methyl substituents, as in the primary amine **29** or when they were in a cyclized form, for example as in the pyrrolidinosqualene **31**. It is noteworthy that, in contrast to pyrrolidinosqualene, derivatives bearing a six-membered ring, such as the azasqualene piperidine **32** or morpholine **33**, retained most of the activity (Figure 6).

3. The inhibition of cyclases by 2-aza-2,3-dihydrosqualene N-oxide **34** and the diethyl derivative **35** seemed favourable to the hypothesis that the oxirane ring opening of SO can be viewed as an SN_2-like reaction, as suggested by Van Tamelen (22,23). Indeed the strong dipolar moments apparently presented by these compounds could show some similarity with the dipolar character in the C-2 oxygen bond appearing during the opening of the oxirane ring. On the other hand, the role of the positively charged species shown by the series of azasqualenes seems to be more favourable to the SN_1 character in the opening of SO. In agreement with this idea, we recalled that the cyclization of a series of SO analogues was partially affected when the natural substrate SO which gives the more stable tertiary carbocation was substituted by the nor or the bis-nor derivative, giving a less stable secondary or primary carbocation.

4. The more or less lipophilic or amphiphilic character of the azasqualene derivatives could be important in achieving the right structure for best activity. Thus, modifying the azasqualene skeleton by saturating the double bond system **44-48,52-54**, we obtained diminished inhibitory activity in all types of SO cyclases tested (34). When the squalene skeleton was made more polar by introducing additional diethyl amine groups **49**, trialkyl ammonium groups **50** or N,N-dialkyl N-oxide groups **51**, the biological activity was maintained (Figure 7).

5. It is known that squalene 2,3;22,23-dioxide, accumulated in the medium is then converted by SO cyclase to 24(S),25-epoxylanosterol, which is further processed to 24(S),25-epoxycholesterol. This latter compound then inhibits HMG-CoA reductase. So we planed the synthesis of azasqualenoids **55,56** which possess an epoxidic group at the other end of the molecule (Figure 8) (35). In a rat microsomal system, we generally found that compounds lacking the epoxidic group were less active and that the N-oxides were more active than the corresponding amines. On the contrary, azasqualene **23** was the most active compound of the series when pig liver SO cyclase was tested; thus there are species-specific differences between rat liver and pig liver microsomal systems. Changing from a microsomal to a solubilized system brings improved activity. We speculate that the positive charge of azasqualenes could be responsible for some interactions with some negative charged compounds of the membrane which are not present in the solubilized system.

6. The in vivo effect of azasqualene derivatives in animals, higher plants and yeasts correlated well with the mimic of the carbocationic character of the intermediates involved during the cyclization of SO, as well as in other enzymes involved in the biosynthesis of sterols. In 3T3 fibroblasts, an IC_{50} = 0.3 μM was calculated when the cells where preincubated with



38 $R_1=R_2=Me,\ R_3=H$

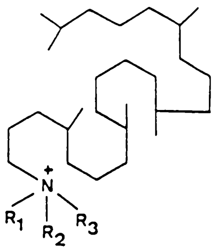
39 $R_1=R_2=Et,\ R_3=H$

40 $R_1=R_2=R_3=Me$

41 $R_1=R_2=Et,\ R_3=Me$

42 $R_1=R_2=Me,\ R_3=O^-$

43 $R_1=R_2=Et,\ R_3=O^-$



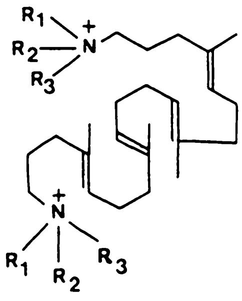
44 $R_1=R_2=Me,\ R_3=H$

45 $R_1=R_2=Et,\ R_3=H$

46 $R_1=R_2=Me,\ R_3=O^-$

47 $R_1=R_2=Et,\ R_3=O^-$

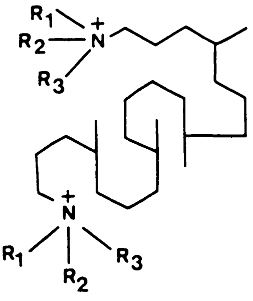
48 $R_1=R_2=Et,\ R_3=Me$



49 $R_1=R_2=Et,\ R_3=H$

50 $R_1=R_2=Et,\ R_3=Me$

51 $R_1=R_2=Et,\ R_3=O^-$

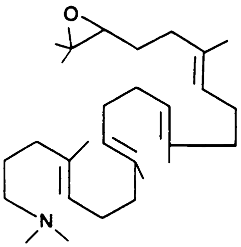


52 $R_1=R_2=Et,\ R_3=H$

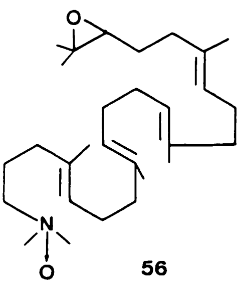
53 $R_1=R_2=Et,\ R_3=Me$

54 $R_1=R_2=Et,\ R_3=O^-$

Fig. 7 Structures of dodecylamine, azasqualene and bis-squalenoid derivatives.



55



56

Fig. 8 Structures of epoxyazasqualene and its N-oxide.

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N,N-diethylazasqualene **25** or its N-oxide **35** (36). The inhibition was correlated with an intracellular accumulation of [^{14}C]-2,3-oxidosqualene and [^{14}C]-2,3;22,23-dioxidosqualene, indicating that the cyclase was indeed an intracellular target of the drug. However, a secondary enzymatic target of azasqualenes proved to be desmosterol Δ^{24} reductase, an enzyme characterized by an intermediate carbocation. We note that desmosterol Δ^{24} reduction can be effected by other ammonium containing molecules such as U-18666A and the 25-azasterol series.

In higher plants (bramble cell suspension cultures) both 2-aza-2,3-dihydrosqualene **23** and the N-oxide derivative **34** inhibited cell growth (IC_{50} of 11 μM and 21 μM respectively) leading to accumulation of SO, while no 2,3;22,23-dioxidosqualene was detectable (37). Azasqualenes also led to a strong accumulation of squalene and Δ^8 sterols, suggesting that they also inhibit squalene epoxidase and Δ^8-Δ^7 isomerase. This latter enzyme catalysed the Δ^7-Δ^8 isomerization of sterols through a C-8 carbocationic intermediate. 2-aza-2,3-dihydrosqualene, but not the corresponding N-oxide is also able to interfere with carotenoid biosynthesis at the lycopene cyclization step, which again seems to involve a carbocationic intermediate (38).

The high specificity shown by azasqualenes and their derivatives towards SO cyclase of yeast has been shown by studying the effect of these compounds on non-saponifiable lipid content during cell growth (39). As shown in Figure 9, growth inhibition by squalene derivatives **25,35,49,51** was higher than that by dodecylamine derivatives **39,43.** The influence of dodecylamine and azasqualene derivatives on sterol biosynthesis was evaluated by incorporation of [^{14}C]-acetate in non-saponifiable lipids. All compounds tested showed a dose-dependent decrease of labelled incorporation into 4,4-desmethyl sterols, and even in the case of azasqualene derivatives, such a decrease corresponded to an increase of radioactive squalene oxide. Among the compounds tested, N,N-diethylazasqualene **25** showed the best correlation between activity on SO cyclase in Saccharomyces cerevisiae microsomal preparations (IC_{50} being 12.5 μM) and inhibition of yeast growth (MIC being 60 μM). In contrast to what was observed in bramble cell cultures (37), 3T3 fibroblasts (36), and Candida albicans cells (40), the squalene epoxidase of S. cerevisiae cells seemed unaffected by azasqualenes.

Most of the compounds known in the literature as inhibitors of SO cyclases are characterized by a basic framework constituted of a nitrogen group linked to a hydrophobic carrier. Among these, one could mention compounds such as N-dodecylimidazole **57**, 2,3-iminosqualene **58**, AMO 1618 **59** and chloroquine **60** (Figure 10).

We showed that 3β-[2-(diethylamino)ethoxy]androst-5-en-17-one (U-18666A) **61** and its dimethyl derivative, confirmed to be potent inhibitors of desmosterol reductase and lanosterol cyclase, as well as N-dodecylimidazole **57**, a potent inhibitor of rat liver microsomal cyclase or epoxidase, were equally active against SO cyclase from higher plants, liver and yeast (33,41-46). We also showed that N-dodecylimidazole may act by a different mechanism compared to the HEI analogues, because by shifting the alkyl chain to position 2 of the imidazole ring, the SO cyclase inhibition activity was completely eliminated. Moreover both AMO 1618 **59** and chloroquine **60** which were described as effecting the biosynthesis of sterols at the cyclization step (47,48), in a whole cell system or in a cell free extract, were

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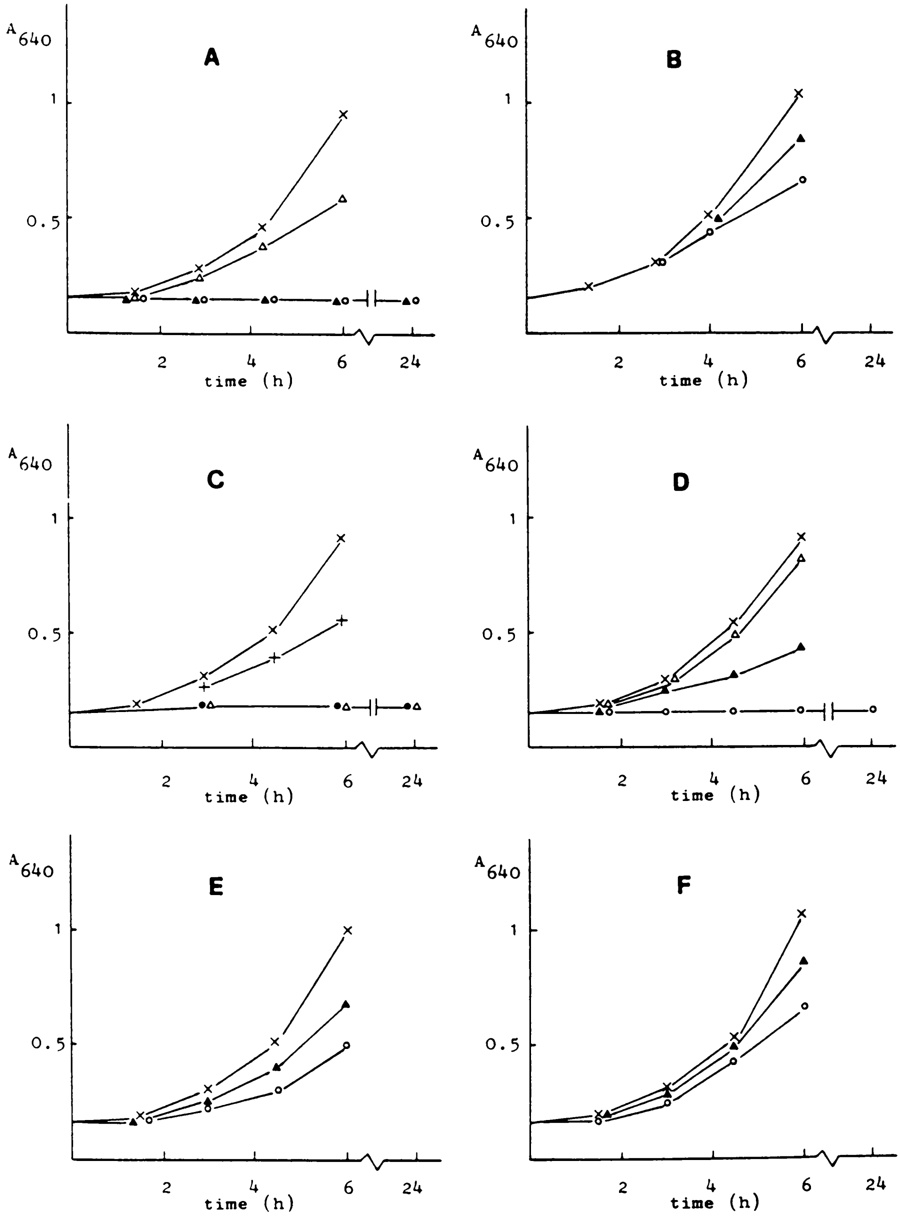

Fig. 9 Effect of azasqualenes and dodecylamine derivatives on the growth of S. cerevisiae: A:25; B:35; C:49; D:51; E:39; F:43.

completely inactive when tested in our system (SO cyclase from rat liver and pea seedling) (33).

2,3-iminosqualene **58** showed strong similarities to azasqualenes in inhibition of microsomal SO cyclase from peas, bramble cells, rat liver and yeast (33,49-51) (Figure 10). Squalene N-methyloxaziridine **62**, similar in structure and electron distribution to both 2,3-iminosqualene **58** and 2-aza-2,3-dihydrosqualene **23**, also strongly inhibited microsomal SO cyclase from rat liver and yeast (I_{50} = 1.5 μM and 2.5 μM respectively) (52) (Figure 11). Oxaziridine **62**, which is the most active inhibitor among the azasqualene series, fitted well with the suggested model of the enzymatic transition state, since either the O or the N atom of the oxaziridine ring may undergo protonation by the acidic site of the enzyme. It is interesting to observe that both squalene aminal hydroperoxide **64** or squalene N-methylamide **65** and squalene N-methylimine **63** poorly inhibited SO cyclase in rat liver or yeast microsomes, but they strongly inhibited cholesterol biosynthesis in 3T3 fibroblasts. Thus, they behave as prodrugs, in that are probably transformed by the enzyme or by cellular metabolism into active products.

Seeking new HEI intermediates of the different carbocations developed during the cyclization of SO, we then focused our attention on the C-8 bicyclic carbocation, postulated as an intermediate.

The Benveniste group, trying to mimic this HEI by a series of azadecalines, found that these compounds were potent inhibitors of cycloeucalenol-obtusifoliol isomerase (53-55), whereas they failed to inhibit the cyclases of higher plants and rat liver. However, when these compounds bore an isoprenoid-like N-substituent, as in **66**, they became excellent inhibitors of both SO lanosterol cyclase from rat liver microsomes and cycloartenol cyclase in microsomes from maize seedlings and bramble cells (Figure 12); however they failed to inhibit the SO β-amyrin cyclase from bramble cells and pea cotyledons (56,57). In addition, these compounds were also very strong inhibitors of Δ^8-Δ^7 sterol isomerase and of cycloeucalenol obtusifoliol isomerase in a cell-free extract from maize seedlings and suspended cultures of bramble cells.

We believed that an acyclic squalenoid molecule bearing a nitrogen atom at the pro C-8 position could behave as a very potent and specific inhibitor of SO cyclase in different tissues. For this reason we planed the total synthesis of both (6E) and (6Z)-10-azasqualene 2,3-epoxide **67**,**68** and checked their preliminary biological activity in animal tissues and yeast (Fig. 12).

The biological results showed that the two isomers differed greatly in their inhibition of SO cyclase, as expected. Isomer E **67**, the carbocation analogue, had an I_{50} of 5 μM on SO cyclase from rat liver microsomes, while isomer Z **68** was practically inactive. On rat liver squalene epoxidase, the two isomers were practically inactive. Tests on SO cyclase of yeast microsomes also showed good selectivity. Isomer E had an approximate I_{50} of 6 μM on S. cerevisiae, while on C. albicans microsomes, the value was about 1.5 μM. Isomer Z was almost inactive on SO cyclase of microsomes of both yeasts.

These preliminary results indicate that we reached three main objectives: 1) only the rationally designed geometrical isomer proved to be active; 2) it had selective activity towards C. albicans; 3) (6E)-10-azasqualene 2,3-epoxide, differently from many SO cyclase inhibitors, had negligible activity towards squalene epoxidase.

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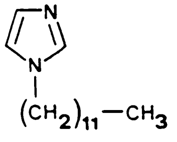

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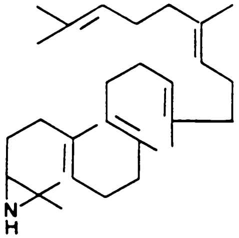

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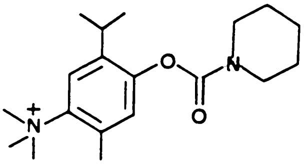

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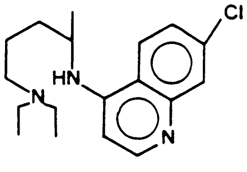

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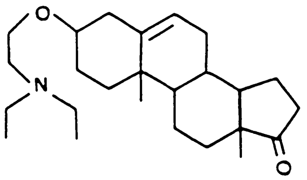
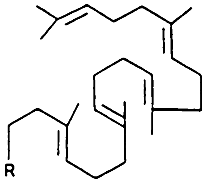
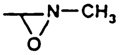


61

Fig.10 Structures of N-dodecylimidazole, 2,3-iminosqualene, AMO 1618, chloroquine and U-18666A.



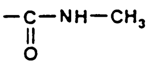
62 R=



63 R= $-CH=N-CH_3$

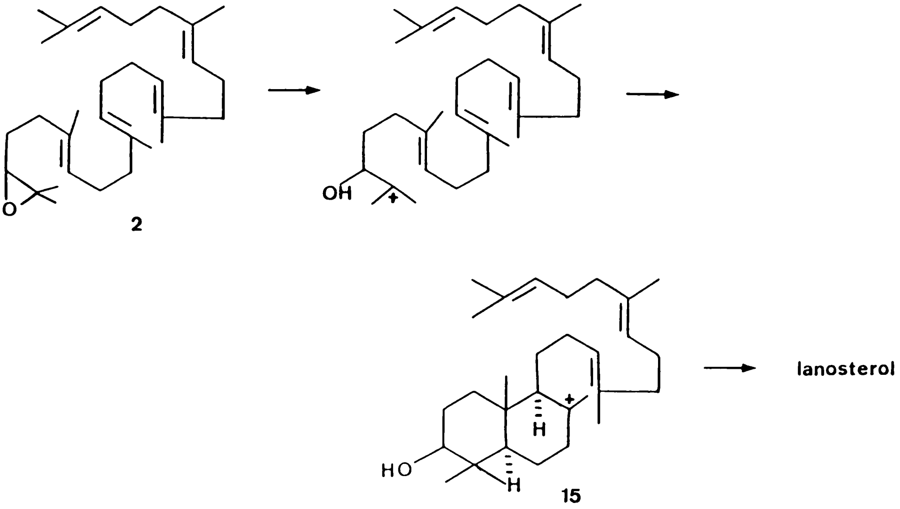
64 R= $-CH(OOH)-NH-CH_3$

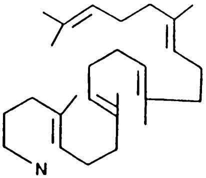
65 R=



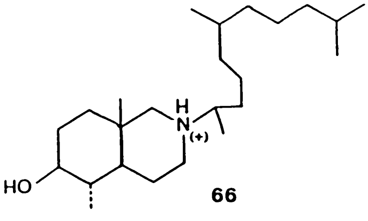
$-\overset{O}{\overset{\|}{C}}-NH-CH_3$

Fig.11 Structures of squalene N-methyloxaziridine and derivatives.

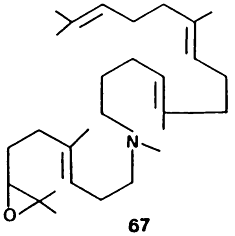




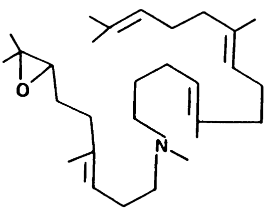
23



66



67



68

Fig.12 Structures of Pro C-2 and Pro C-8 high energy intermediates: azasqualene, azadecaline, 6E and 6Z-10-azasqualene 2,3-epoxide.

Squalene Epoxidase Inhibitors

Squalene epoxidase, an enzyme responsible for the stereospecific oxidation of squalene **1** to 2,3-oxidosqualene **2** (Figure 2), is composed of a terminal oxidase, a flavin-containing enzyme different from cytochrome P-450 isoenzymes, and a flavoprotein identical to NADPH cytochrome P-450 reductase (58,59).

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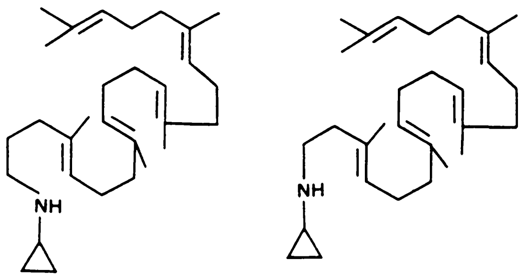
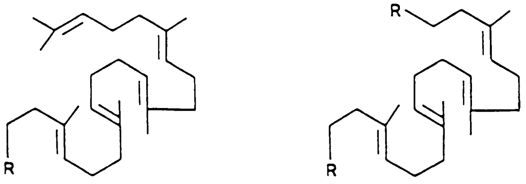


69 **70**



71 R= $-C\equiv CH$
72 R= $-C\equiv C-CH_3$
73 R= $-CH=C=CH-CH_3$

74 R= $-C\equiv CH$
75 R= $-C\equiv C-CH_3$
76 R= $-CH=C=CH-CH_3$

Fig.13 Structures of squalenoid cyclopropylamines, acetylenes and allenes.

Comparison of enzymatic incubations with different squalene analogues showed that the epoxidation could tolerate considerable variations at the epoxiding site (60). This is in contrast with SO cyclization, where only small modifications at the substrate terminal olefin are tolerated. Moreover, squalene epoxidase required a substrate that had to approximate the size and the shape of squalene.

Some azasqualenes, such as 2-aza-2,3-dihydrosqualene **23** were found to be inhibitors of squalene epoxidase from C. albicans and rat liver, with an I_{50} of 33 μM and 2.4 μM respectively, but not of the enzyme from S. cerevisiae (40). It is probable that 2-aza-2,3-dihydrosqualene behaved similarly to squalenoid cyclopropylamines **69,70** sinthesized by Prestwich (Figure 13) (61), which were found to be potent inhibitors of pig liver squalene epoxidase and were suggested to act as slow tight-binding inhibitors.

Since many flavin-linked enzymes proved to be readily inactivated by β,γ-acetylenic compounds or by allenic derivatives through the formation of high reactive intermediates which could be attacked by an enzyme nucleophile, we prepared a series of mono- and bifunctional analogues of squalene **71-76** containing an acetylenic function, such as squalenoid acetylene, methylacetylene, bis-acetylene, bis-methylacetylene or an allenic function, such as methylallene or bis-methylallene (Figure 13) (62).

Inhibition by these compounds was shown to be specific towards microsomal squalene epoxidase associated with rat liver microsomes, since they did not inhibit a cytochrome P-450 reductase system from rat liver microsomes (aminopyrine demethylase). The results showed that the squalenoid molecule which is bifunctional and carries an allenic function as **76** is the best inhibitor of squalene epoxidase (IC_{50} = 50 μM). This could suggest that the enzymatic epoxidation, as exemplified by the epoxidation of 2,3-oxidosqualene to 2,3;22,23-dioxidosqualene may occur symmetrically on the two terminal isoprenic units of squalene.

Similar acetylenic or allenic analogues were synthesized later by Prestwich (63) who found no activity for the compounds as inhibitors of squalene epoxidase from pig liver. This apparently contradictory result was interpreted as a species-specific dependence of the enzyme.

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Chapter 14

Squalene Epoxidase Inhibitors

Structural Determinants for Activity and Selectivity of Allylamines and Related Compounds

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The allylamine antifungal agents naftifine, terbinafine and numerous related compounds act by inhibiting fungal squalene epoxidase but have a much weaker effect on the equivalent enzyme from mammalian sources. Terbinafine is a reversible non-competitive inhibitor of squalene epoxidase from *Candida* (Ki=30 nM) and a reversible competitive inhibitor of the rat liver microsomal enzyme (Ki=77 µM). The structurally related NB-598 has reversed selectivity with potent inhibition of mammalian epoxidase and no effect on the fungal enzyme. The structural features required for epoxidase inhibition and selectivity have been examined. The terbinafine side-chain appears to be optimal for inhibition of both enzymes while modifications of the naphthalene ring can lead to either improved antifungal activity or to selectivity for the mammalian epoxidase.

Squalene epoxidase plays a key role in the biosynthetic sequence from acetate to sterols and is the first enzyme in the pathway which requires molecular oxygen. The presence of the 2,3-epoxide is a pre-condition for subsequent cyclization to the rigid 4-ring sterol backbone. The capability to synthesize sterols is thus limited to aerobic organisms. Squalene epoxidase is a membrane-bound enzyme first described in rat liver microsomes by Yamamoto and Bloch (*1*). Similar enzymes are known to occur in other mammalian tissues and eukaryotic cells, including fungi. The enzymology of squalene epoxidase and its inhibitors has recently been reviewed (*2*). The first specific inhibitor of squalene epoxidase to be identified was the antifungal agent naftifine, compound **1** (*3-5*) which represents the prototype of a separate class of antimycotics, the allylamines (*6*). The biological and clinical properties of these drugs, including the oral antimycotic terbinafine, compound **2** , (*7,8*) are the subject of a recent review (*9*) . An interesting aspect of the allylamines is that they show high selectivity between fungal and mammalian epoxidases (*2*) . More than one thousand derivatives of the

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allylamine type have been synthesized and tested for antifungal activity, permitting extensive study of structure-activity relationships (*10-15*). In this article we review current knowledge concerning the mechanism of squalene epoxidase inhibition by allylamines, and the structural requirements for this inhibition.

Inhibition of Sterol Biosynthesis

The antifungal activity of 1 and 2 appears to result solely from their inhibition of fungal ergosterol biosynthesis, the blockade of squalene epoxidation causing accumulation of squalene and deficiency of ergosterol in treated cells (*5,8,16-18*). The build-up of high intracellular levels of squalene is thought to be responsible for the primary fungicidal action of these drugs (*9,19*). Structurally similar compounds which have been characterised as antifungal agents include SDZ 87-469 (*10,20*), 3, and the benzylamine derivative butenafine, 4, (*21*), both of which are squalene epoxidase inhibitors as described below. The significance of this enzyme as an antifungal target is underlined by the existence of a second class of antifungal squalene epoxidase inhibitors, the thiocarbamates (*22,23*) which show some structural resemblance to 1.

The antifungal allylamines are highly selective for inhibition of the fungal squalene epoxidase and thus have no effect on mammalian cholesterol biosynthesis (*18,24*). However, recently the structurally related compound NB-598 (5) has been reported to be a potent inhibitor of mammalian squalene epoxidase and cholesterol biosynthesis *in vitro* and *in vivo* while having no antifungal activity (*25*).

Measurement of Inhibition. Several experimental systems were used to assess the activity of allylamines as sterol biosynthesis inhibitors, using incorporation of radiolabelled precursors. Three standard methods described below were employed in comparison and selection of compounds, although only compounds of exceptional interest were tested in all three. In the subsequent tables of data, these tests are referred to as "Cells", "Cell-free" and "Epoxidase". Comparison between inhibitory values obtained in whole-cell and cell-free tests is important in assessing the ability of a compound to penetrate the fungal cell envelope, a property essential for antifungal activity. There is generally very good agreement between values from the cell-free and squalene epoxidase tests. The direct enzyme assay was also used for detailed kinetic investigations of selected compounds.

Whole Cell Test. Fungal cells grown to late exponential phase were washed and incubated 2 hours with labelled acetate in a buffered glucose medium (*5,8*). The non-saponifiable lipids were then extracted and analyzed by thin layer chromatography. Fractions corresponding to squalene, 4,4-dimethylsterols, 4-monomethylsterols and ergosterol were counted for radioactivity, usually accounting for about 95% of the total incorporated radioactivity. Inclusion of a squalene epoxidase inhibitor such as terbinafine caused a dose-dependent inhibition of incorporation into the ergosterol and other sterol fractions with a corresponding accumulation of label in the squalene fraction (*5,8,17*). From the dose-response curves, drug concentrations for 50% inhibition (IC-50) could be calculated. All values presented here are from yeast-form cells of the pathogenic dimorphic yeast *Candida albicans*. For selected compounds such as 1, 2 and 3, results have been obtained in a range of filamentous and yeast-like

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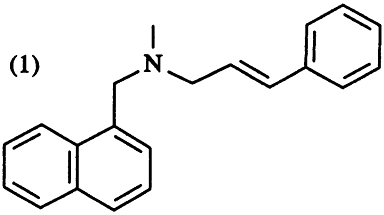

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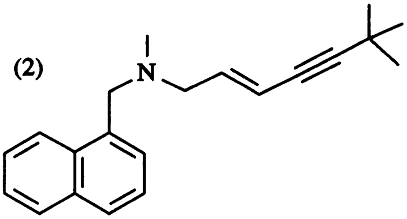

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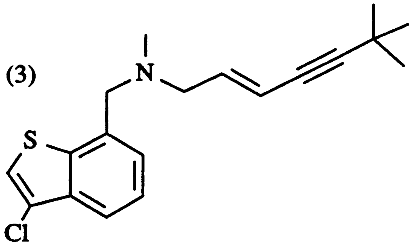

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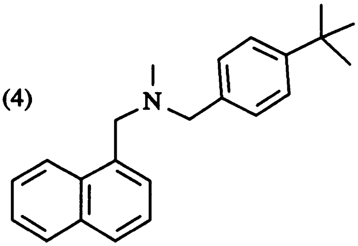

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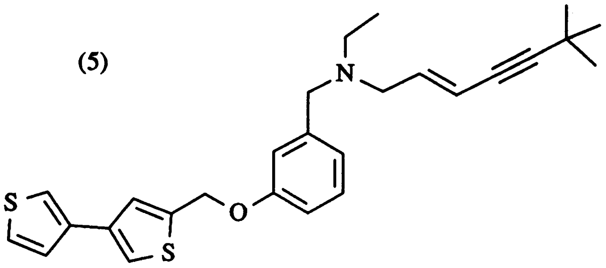

Table I. Selective Inhibition of Fungal Squalene Epoxidase

Compound	I.C.-50 (µM)			
	Candida albicans	*Candida parapsil.*	Rat Liver	Guinea Pig Liver
1	1.1	0.34	144	>100
2	0.03	0.04	77	4
3	0.011	0.02	43	1.2
4	0.045	n.d.	n.d.	23

n.d.: not done

fungi, showing good correlation between inhibition of growth and of sterol biosynthesis (*8,17*).

Cell-free Test. *Candida* cells were disrupted by homogenizing or (for best results) by sphaeroplasting (*26*) and centrifuged at 10,000 g. The resulting cell-free extract incorporated radiolabelled mevalonate into the sterol fractions in the presence of a cocktail of biochemical cofactors (*5,8*). Measurement of IC-50 values was as for the whole-cell system. A very similar system derived from rat liver homogenate was used to investigate effects on mammalian cholesterol biosynthesis (*8,18*).

Squalene Epoxidase Assay. The *Candida* microsomal squalene epoxidase was characterized and optimal assay conditions worked out on the principle of measuring conversion of labelled squalene to squalene epoxide (*24,26,27*). A similar assay was employed for the microsomal epoxidase from rat liver (*24*) and guinea pig liver (*26*).

Specificity of Inhibition. The possibility of allylamines inhibiting steps other than squalene epoxidase in the ergosterol biosynthesis pathway was investigated in a range of cell-free tests using several different labelled sterol precursors (*8,17*). Possible effects on steps distal to squalene epoxide in whole fungal cells were also examined by measuring sterol side-chain methylation which occurs later in the pathway (*28*). No evidence could be found for significant inhibition of any enzyme other than squalene epoxidase, using **1**, **2** and **3** as test compounds. These findings are supported by comparisons of the inhibitory concentrations in different test systems (see below), which indicate that epoxidase inhibition fully accounts for the observed inhibition of cellular ergosterol biosynthesis.

Selective Inhibition of Fungal Squalene Epoxidase.

Squalene epoxidase is a membrane-bound microsomal enzyme requiring molecular oxygen, FAD, and a source of reducing equivalents (NADH or NADPH). The epoxidase is not an enzyme of the cytochrome P-450 superfamily, does not contain heme and is unaffected by known inhibitors of cytochrome P-450 such as carbon monoxide. This is important from a therapeutic point of view, as the allylamines have no tendency to inhibit the P-450 enzymes which have many important functions in the human body (*29*).

Since **2** is also an orally active drug, its effects on both fungal and mammalian epoxidases were characterized. For this purpose, the microsomal epoxidases from *C. albicans* and *C. parapsilosis* were investigated (*24,27*). The rat liver enzyme was the subject of extensive studies by Bloch and coworkers and has been purified (*30*).

Fungal Squalene Epoxidase. Compounds **1** and **2** and related structures cause a concentration-dependent inhibition of the *Candida* epoxidase and complete suppression of activity is attainable (*24*). Kinetic analysis indicates both compounds to be non-competitive with respect to the substrate squalene and the cofactors FAD, NADH and NADPH. Table I shows inhibitory concentrations for **1** to **4**; the potency of these drugs reflects their respective antifungal activities. The inhibition is fully reversible by dilution and is not time-dependent, indicating that covalent modification of the enzyme

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does not occur. Very similar results were obtained with the two *Candida* species. Full kinetic analysis has only been carried out with **1** and **2** but it can be assumed that other allylamines are qualitatively similar in effect. The non-competitive nature of the inhibition is in agreement with the observation that the high squalene concentrations occurring in treated fungi do not lead to reversal of the drug-induced growth arrest. *C. albicans* squalene epoxidase can be solubilized by detergent action but remains equally sensitive to inhibition by **2**, indicating that the inhibition is direct and not dependent on membrane integrity.

Mammalian Squalene Epoxidase. Allylamines **1-3** proved to be very weak inhibitors of the rat liver microsomal enzyme (Table I). Kinetic studies with **2** showed that, in contrast to the fungal enzyme, inhibition of the liver epoxidase is competitive with squalene. Inhibition is reversible and non-competitive with FAD and NAD(P)H. The epoxidase from liver microsomes differs from the fungal enzyme in being almost completely dependent for activity on a soluble cytoplasmic protein factor. Inhibition by **2** was found to be competitive with the soluble cytoplasmic fraction, but not to an extent which would account for the high selectivity with respect to the fungal epoxidase (*24,26*).

Examination of several mammalian squalene epoxidases revealed a considerable variation in their sensitivity to allylamines. The enzyme from guinea pig liver was found to be exceptionally sensitive, although still orders of magnitude less sensitive than the fungal enzyme, and was employed to test a number of compounds (Table I). The reason for this difference in sensitivity is not known, but is clearly not due to differences in binding to the respective soluble cytoplasmic factors (*26*).

All the antifungal allylamines which have been investigated were found to be relatively inactive against the mammalian squalene epoxidase. However, compound **5** with a terbinafine-type sidechain and greatly modified ring structure has recently been reported as a very potent inhibitor of human microsomal squalene epoxidase, with Ki= 0.68 nM (*25*). Interestingly, **5** is also competitive with regard to squalene as in the case of **2**, although with 4 to 5 orders of magnitude difference in potency. The selectivity of **5** is the reverse of that of **2**, the former compound having no antifungal activity (*25*) and no significant effect on ergosterol biosynthesis (Table II), indicating that selectivity is at the enzymatic level.

Mechanism of Epoxidase Inhibition.

The squalene epoxidase system presents a number of possible targets for inhibition, including the terminal oxidase, the reductase enzyme to which it is coupled, the lipid environment and , in the case of the mammalian enzyme, the soluble cytoplasmic factor. The allylamines do not inhibit the reductase and have only a weak interaction with the cytoplasmic factor. Mechanism-based irreversible types of inhibition are ruled out by the kinetic properties of inhibition in both fungal and mammalian systems.

Fungal Enzyme. The non-competitive kinetics and high selectivity render it unlikely that the allylamines are acting as substrate analogues, and indeed their general structure has little in common with that of squalene. It is known that epoxidase activity requires phospholipids and is affected by various fatty acids (*2*). A recently suggested model

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Table II. Selective Sterol Biosynthesis Inhibition by Terbinafine (2) and NB-598 (5)

Compound	I.C.-50 (μM)		
	Candida Cells	*Candida* Cell-free	Rat Liver Epoxidase
2	0.03	0.03	77
5	>100	34	0.004[a]

[a] Value from reference *25*.

(*31*) postulates that the inhibitor binds with relatively low affinity to two separate sites on the epoxidase, the naphthalene ring to the squalene-binding site, and the sidechain to an adjacent lipophilic pocket, resulting in high-affinity entropic binding of the molecule to the enzyme. This concept is compatible with all the available evidence.

The two-site model is supported by experiments with fragments of 2; the sidechain alone has no inhibitory effect on the *Candida* epoxidase at concentrations up to 1 mM. However, naphthalene (6) and 1-methyl derivative (7) are very weak inhibitors (Table III). This activity is lost with addition of the hydrophilic amine group (8, 9), which causes strong polarization of the molecule, but then increases with a tertiary amine and increasing chain length (10-12). Addition of the terminal tertiary butyl group then delivers a 10,000-fold increase in activity (comparing 2 and 12). This suggests the need for a minimum chain length to bridge the two binding sites on the enzyme.

Mammalian Enzyme. Selectivity might be explained by differences in alignment or distance between the two postulated binding sites. The competitive inhibition shown by both 2 and 5 suggests interaction with the substrate-binding site of the mammalian enzyme, weak in the case of 2 and strong in the case of 5. Unlike 2, the extended structure of 5 could provide the possibility of occupying the squalene-binding site with high affinity.

This concept of selective inhibition is of course highly speculative and will require further detailed studies of the respective enzymes for confirmation.

Structural Determinants for Epoxidase Inhibition.

Initial studies (*15*) of structure-activity relationships of 1 and its derivatives, based on antifungal activity, indicated the specific structural requirements for this activity. These included the 1-substituted naphthalene and tertiary allylamine groups, while considerable modification of the sidechain was permitted.

Modification of Sidechain. As shown in Table IV, variation of the sidechain of 1 can lead to greatly increased epoxidase inhibitory activity. The rigid analogue 13 is interesting in showing stereospecificity at the chiral centre, the *R*-enantiomer having

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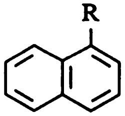
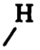
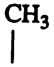
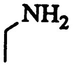
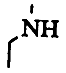
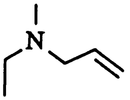
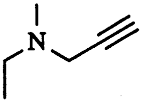
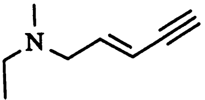
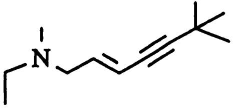
TABLE III. Inhibition of *C. albicans* Squalene Epoxidase by Naphthalene Derivatives

		**Squalene Epoxidase Inhibition(%)**		
Nr.	**R**	**10^{-5}M**	**10^{-4}M**	**10^{-3}M**
6		11	16	84
7		7	0	85
8		0	0	0
9		-27	0	26
10		0	0	100
11		33	63	100
12		0	37	84
2[a]		100	100	100

[a] IC_{50}= 0.03 µM

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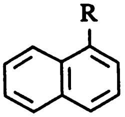
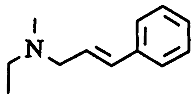
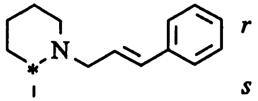
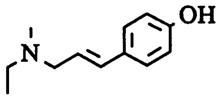
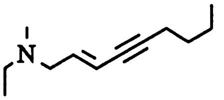
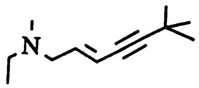
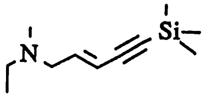
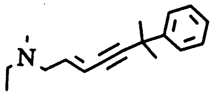
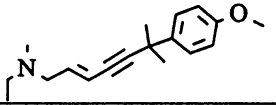
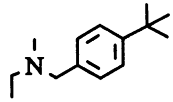
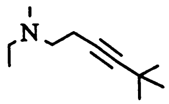
TABLE IV. Effect of Sidechain Variation on Inhibition of *C. albicans* Sterol Biosynthesis and Squalene Epoxidase

		I.C.$_{50}$ (µM)		
Nr.	**R**	**Cells**	**Cell-free**	**Epoxidase**
1		1.08	0.59	0.93
13a		0.36	0.14	0.11
13b		4.95	2.47	n.d.
14		>100	38.8	n.d.
15		0.21	0.10	n.d.
2		0.03	0.03	0.03
16		0.03	0.03	n.d.
17		n.d.	n.d.	0.04
18		n.d.	n.d.	0.09
4		0.14	0.05	0.05
19		n.d	n.d	0.05

10-fold superior activity, which also correlates with antifungal activity (*14*). As previously shown in Table III, the length of the sidechain is important, and the enyne sidechain of 2 appears to be optimal in this respect. Further extension (**17**, **18**) gives no further increase in activity. The benzylamine (**4**) and homopropargylamine (**19**) sidechains are of similar length to the enyne and have comparable activity. A bulky terminal group appears to be beneficial (compare **2** and **15**), while addition of a polar group (**14**, **18**) is unfavorable, in agreement with the two-site model of inhibition. The trimethylsilyl derivative **16** has activity equivalent to that of **2**, which is also translated into antimycotic activity (*13*).

Allylamine Double Bond. It was previously known from antifungal activities that the *trans* configuration of the double bond was required for activity, the *cis* isomer being much less active (*13*). This specificity clearly operates at the enzymatic level, seen in comparison of **2** and its *cis* isomer **20** (Table V). The *cis* configuration also appears to have an adverse effect on cell penetration. Fluorination at the double bond either has little effect (**21**), or leads to reduction in activity (**22**). Although the configuration is important, the double bond itself is not required for epoxidase inhibition, as demonstrated by the activity of **19** in Table IV.

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Allylamine Nitrogen. Removal of the *N*-methyl group (**23**) leads to loss of activity, but substitution with ethyl (**24**) does not increase activity above that of **2**. The low but significant epoxidase inhibition shown by the oxa analogue **25** indicates that the amino nitrogen is not strictly required for activity. Further clarification of this point was obtained by synthesis (*11*) of a series of carbon analogues of **2**, compounds **26**-**33**. The results (Table VI) demonstrate inhibitory activity to be related to similar steric requirements as in **2**, that is, methyl substituent at the carbon replacing the nitrogen, and *trans* configuration of the double bond. The specific attributes of the nitrogen atom are not required. The active compounds **27** and **32** showed antifungal activity against dermatophytes only (*11*). Comparing the values for inhibition in whole cells and cell-free extracts (Table VI), it is clear that presence of the allylamine nitrogen is important for penetration of the compounds through the fungal cell envelope. The surprisingly good activity of the cumulenes **31**-**33** gives further support for the concept of the sidechain acting as a rigid bridge between two separate binding sites.

Modification of Naphthalene Ring. The size of the ring structure is critical for inhibitory activity, and the monocyclic compounds **34**-**36** show only weak activity (Table VII). Within the naphthalene series, 4-substitution leads to reduced activity (compound **37**), while substitution at some other positions is permitted but without improving activity (compounds **38** and **39**). Naphthalene can be replaced by benzo[*b*]thiophene with equivalent activity (compounds **40** and **41**) but enlargement of the ring to a dibenzothiophene (**42**) leads to loss of activity. The 3-chloro-7-benzo[*b*]thienyl compound **3** is significantly higher in activity (Table VII), and is in fact the most potent inhibitor of fungal squalene epoxidase which has been found up to now, correlating with its very high antifungal efficacy (*10,20*).

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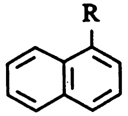
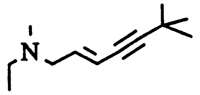
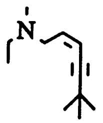
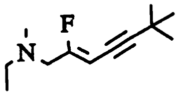
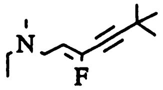
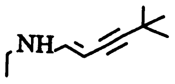
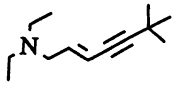
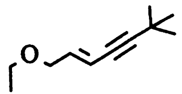
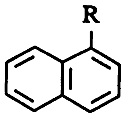
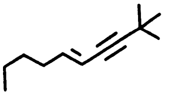
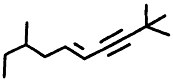
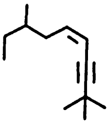
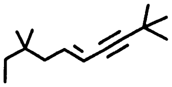
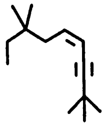
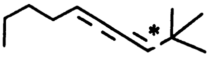
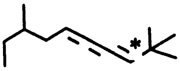
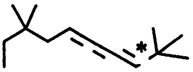
TABLE V. Effect of Variation in Allylamine Group on Inhibition of *C. albicans* Sterol Biosynthesis and Squalene Epoxidase

		$I.C._{50}$ (µM)		
Nr.	**R**	**Cells**	**Cell-free**	**Epoxidase**
2		0.03	0.03	0.03
20		2.44	0.64	n.d.
21		n.d.	n.d.	0.05
22		n.d.	n.d.	0.42
23		0.32	0.32	n.d.
24		0.04	0.03	n.d.
25		n.d.	n.d.	2.16

n.d. = not done

TABLE VI. Inhibition of *C. albicans* Ergosterol Biosynthesis by Carbon Analogs of Allylamines

		I.C.$_{50}$ (μM)	
Nr.	**R**	**Cells**	**Cell-free**
26		>30	16.3
27		2.69	0.24
28		>30	2.48
29		n.d.	>30
30		n.d.	>30
31		n.d.	0.47
32		0.34	0.10
33		>30	0.56

**E*:*Z* - 1:1

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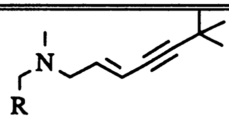
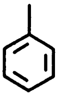
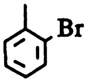
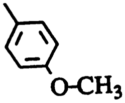
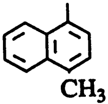
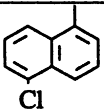
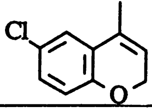
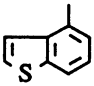
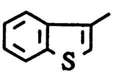
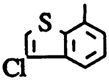
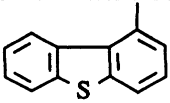
TABLE VII. Effect of Ring Modification on Inhibition of *C. albicans* Sterol Biosynthesis and Squalene Epoxidase

		I.C.$_{50}$ (μM)		
Nr	**R**	**Cells**	**Cell-free**	**Epoxidase**
34		n.d.	n.d.	3.87
35		1.56	1.56	n.d
36		184	n.d.	47.9
37		0.43	0.21	n.d.
38		n.d.	0.02	n.d.
39		0.03	0.03	n.d.
40		0.03	0.03	n.d.
41		0.03	0.03	n.d.
3		0.011	0.007	0.011
42		n.d.	n.d.	0.37

n.d. = not done

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Chapter 15

Inhibition of Sterol Metabolism in Insects and Nematodes

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Highlights of research on inhibition of sterol metabolism in insects and nematodes is summarized and discussed. Steroid metabolism in insects and nematodes presents a unique area of biochemical differences from vertebrates and plants, since neither insects nor nematodes are able to biosynthesize the steroid nucleus. Both insects and nematodes rely on dietary sources of sterol for normal growth and development and are capable of considerably altering their dietary sterols, particularly C_{28} and C_{29} phytosterols, to produce cholesterol as well as a large number of other sterols. Further, cholesterol is essential for the production of molting hormones (ecdysteroids) in most insects. Thus, the disruption of uptake, transport, or metabolism of dietary sterols in insects or nematodes could be fatal to the organism and could lead to new, specific control technology. A variety of compounds have been developed that effectively inhibit sterol metabolism in insects and nematodes and demonstrate that this is a vulnerable area of biochemistry in these organisms to exploit.

Steroid metabolism is an extremely important area of biochemical difference between insects and nematodes and many other organisms, particularly vertebrates, that might be exploited in the development of new pest control strategies (*1,2*). Neither insects nor nematodes are able to biosynthesize the steroid nucleus and require a dietary source of sterol for normal growth, development, and reproduction (*3,4*). It is of interest to note that this dietary requirement for sterol represents the only proven difference in nutritional requirements between insects and mammals (*5*). Cholesterol generally satisfies the requirement for both insects and nematodes, but little or no cholesterol is available in the natural diet of many phytophagous insects or plant-parasitic nematodes. Dealkylation at C-24 of C_{28} and C_{29} phytosterols enables many of these insects and nematodes to produce cholesterol (Figure 1) (*1,6*). The major ecdysteroids (molting hormones) in insects are C_{27}

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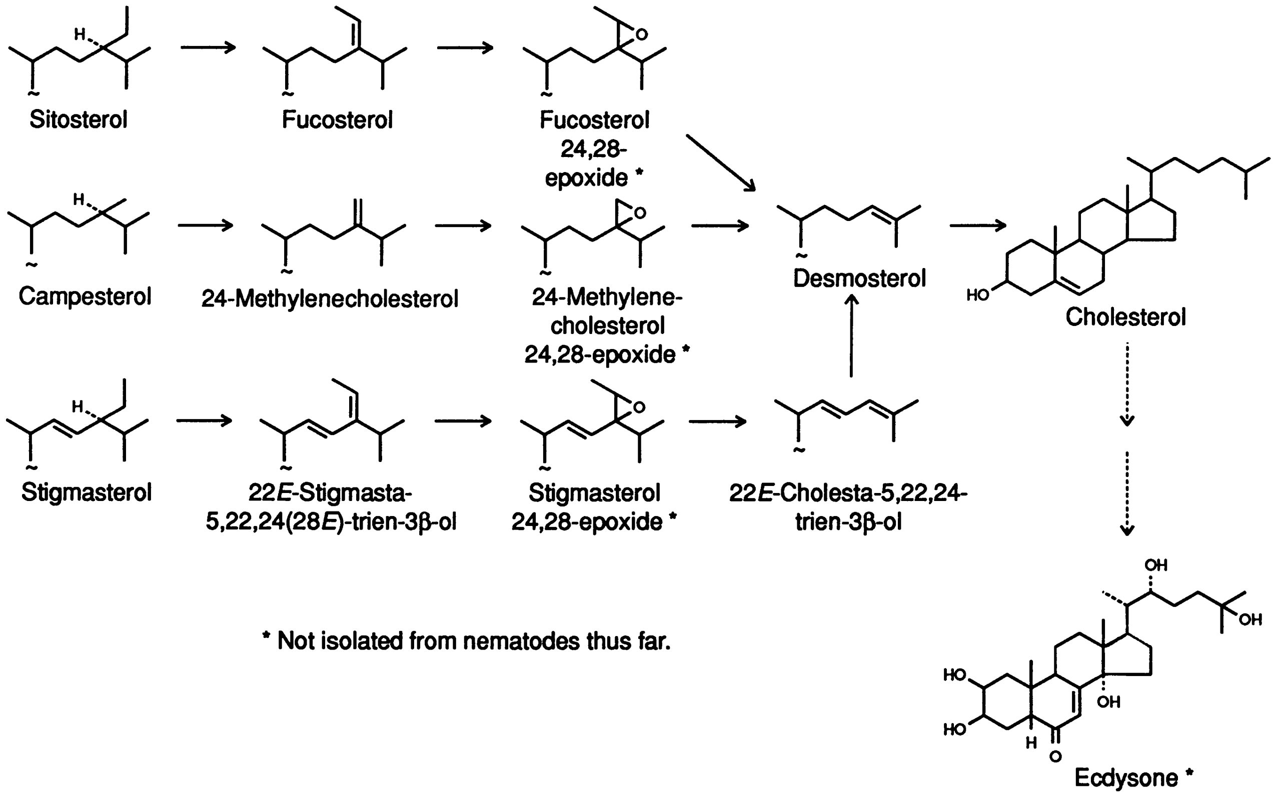
Sitosterol
Fucosterol
Fucosterol 24,28-epoxide *
Campesterol
24-Methylenecholesterol
24-Methylene-cholesterol 24,28-epoxide *
Desmosterol
Cholesterol
Stigmasterol
22*E*-Stigmasta-5,22,24(28*E*)-trien-3β-ol
Stigmasterol 24,28-epoxide *
22*E*-Cholesta-5,22,24-trien-3β-ol
Ecdysone *
H
O
HO
OH

* Not isolated from nematodes thus far.

Figure 1. Generalized metabolic pathways of sterol metabolism in insects and nematodes

steroids produced from cholesterol (*7*). In addition to involvement in regulating the molting process, ecdysteroids have been shown to have many physiological functions in insects (*8*). Thus steroid metabolism presents two unique target areas that might be exploited by (i) disrupting cholesterol production through the inhibition of dealkylation and conversion of C_{28} and C_{29} phytosterols to cholesterol in those insects or nematodes that are dependent on this mechanism, and thus reducing the amount of available molting hormone precursor, or (ii) blocking any of the several biosynthetic steps in insects between cholesterol and ecdysone. Successful disruption of either of these biochemical sequences could effectively block the molting process in the early larval stages, which would be highly desirable with many of the economically important phytophagous pest species, or interfere with the reproductive process. In-depth studies of the biochemical pathways of utilization and metabolism of C_{28} and C_{29} phytosterols and ecdysteroid occurrence and metabolism in insects have yielded considerable basic information and made possible the development of new, selective model compounds that disrupt steroid metabolism, the molting cycle, and reproduction in certain insects and other pests. Although it has not been possible to demonstrate ecdysteroid biosynthesis and function in nematodes, many other aspects of steroid metabolism are similar to what is found in most phytophagous and omnivorous insect species. These similarities have made it possible to demonstrate that sterol metabolism and development of certain plant and animal parasitic as well as free-living nematodes could be disrupted by many of the steroid metabolism inhibitors developed in insect studies. We will focus our discussion of inhibitors of steroid metabolism on the studies with insects and nematodes that have shown fairly direct effects on metabolic pathways.

Inhibition of Insect Sterol Biosynthesis

Insect Steroid Biochemistry. Continued loss of insecticides from our chemical arsenal through development of resistance or environmental concerns has made it necessary to continue to search for alternate selective, biorational insect control technology. The uniqueness of steroid biochemistry in insects continues to provide a viable area of significant biochemical difference that might prove to be useful in this search.

Detailed studies of the metabolism of a number of C_{28} and C_{29} phytosterols, which comprise the major percentage of dietary sterols encountered by phytophagous insects, have revealed that certain biochemical pathways (Figure 1) involved in the dealkylation and conversion of these sterols to cholesterol are common to many species of phytophagous and omnivorous species, although not to all of them (*3,9*). A number of intermediates in these pathways are known and some enzyme systems are available to study specific effects of certain inhibitors. The scheme shown in Figure 1 is common to many insects that dealkylate phytosterols. However, there are variations among insects that can dealkylate, but that is an area beyond the scope of this discussion (*3*). It is interesting to note that in the conversion of each of the most ubiquitous phytosterols (sitosterol, campesterol, and stigmasterol) to cholesterol, a 24,28-double bond intermediate is initially formed, followed by oxidation to an epoxide, and subsequent conversion

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to desmosterol (Δ^{24}) which is the final intermediate common to each of these pathways.

The conversion of cholesterol to ecdysone has been studied extensively, but major gaps still remain in our knowledge of several early steps in this pathway (*10*). Although considerable evidence exists indicating that 7-dehydrocholesterol is an early intermediate following cholesterol, the pathway from there to 2,22,25-trideoxyecdysone as the next logical known intermediate is not well understood. Studies from several laboratories, summarized by Rees (*10*), provide strong support that the subsequent series of hydroxylations to produce ecdysone occur in the order: C-25, C-22, C-2. Finally, a C-20-hydroxylation produces 20-hydroxyecdysone, the active form of ecdysteroid in most species studied.

Azasteroids and Amines and Amides. After the discovery that desmosterol is the terminal intermediate in the dealkylation and conversion of C_{28} and C_{29} phytosterols to cholesterol in the tobacco hornworm, *Manduca sexta*, and a number of other phytophagous and omnivorous species of insects (*11,12*), we found that certain vertebrate hypocholesterolemic agents effectively inhibited the conversion of phytosterols to cholesterol at the Δ^{24}-sterol reductase level and disrupted larval development (*12,13*). This was the first example of inhibition of a specific steroid metabolism enzyme in insects. In these inhibited larvae, the amount of cholesterol produced was greatly reduced, and desmosterol accumulated. In addition, there was evidence that some of these inhibitors affected aspects of steroid metabolism in insects other than the Δ^{24}-sterol reductase (*12*). First we studied triparanol and a group of more than 20 azasteroids, including 22,25-diazacholesterol and related compounds. Structure-activity studies provided information that enabled the design and synthesis of several new, structurally simple, much more potent azasteroids of which 25-azacholesterol methyl ester, 25-azacholestane and 25-azacoprostane were the most active. Each of these three compounds had nearly 1,000 times the activity of the first azasteroids tested (*14*). These were lethal at a concentration of 1 ppm or less in the diet or medium of *Manduca*, the fall armyworm, *Spodoptera frugiperda*, and yellowfever mosquito, *Aedes aegypti*. Depending on concentration used, these azasteroids inhibited the Δ^{24}-sterol reductase enzyme, disrupted early larval molts, or interfered with pupation or adult emergence.

The azasteroids provided valuable tools in the in-depth investigation of steroid metabolism in insects as their inclusion in the diet in combination with phytosterols brings about the accumulation of intermediates in higher than normal quantities (*14*). However, their cost of synthesis and steroidal nature would limit their development for use as insect control agents. Based on information from structure-activity relationship studies with the azasteroids, nonsteroidal compounds were designed that might have the same types of biological and biochemical activities. The dimethylamine functional group was critical for optimum activity with the azasteroids, and this group was incorporated into a series of new compounds. Opening the A ring to form seco-steroids did not decrease the activity and indicated that the intact steroid nucleus was not necessary (*15*). Bicyclic compounds with only the steroidal C and D rings and 25-azacholesterol side chain were less potent, but still, at sufficiently high concentrations, inhibited the Δ^{24}-reductase and larval development of insects such as *Manduca* (*16*). Then it was discovered that structurally simple branched- and straight-chain alkyl amines (e.g.

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N,N-dimethyldodecanamine) and certain related amides were considerably more potent than the bicyclic amines and produced all the characteristic effects on development and steroid metabolism caused by the azasteroids (*16*). These simple compounds were not as active as the most potent azasteroids, but some were effective in the parts per million range, particularly against the yellowfever mosquito and the confused flour beetle, *Tribolium confusum*. Also, nonspecific inhibition in a number of species of insects in studies with azasteroids and amines has been reported (*1*).

Although the branched- and straight-chain alkyl amines have not provided any compounds of sufficient potency for immediate use against any of our insect pest problems, studies with other biological systems have provided exciting information on the effectiveness of some of these inhibitors against other important agricultural pests. These cooperative studies were arranged to determine if we could take advantage of the simplicity of structure, low toxicity and cost, and commercial availability of some of these compounds. Several biological activities with considerable potential were discovered as a result of this research, the most significant of which were (i) high nematicidal activity against certain plant-parasitic nematodes (discussed in detail later in this chapter), (ii) miticidal activity against psoroptic scabies mites of livestock (*17,18*), and (iii) antimicrobial activity against the bacteria responsible for the major part of the mastitis problem of the dairy industry (*19*). As a result of the latter research, *N,N*-dimethyldodecanamine is used commercially as a teat dip to combat mastitis.

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Other Side-Chain Metabolism Inhibitors. Hikino *et al.* found that another group of nitrogenous steroids specifically inhibited sterol metabolism and overall development in the silkworm, *Bombyx mori* (*20*). Three compounds, including 3β-(β,β-dimethylaminoethoxy)-androstene-5-en-17-one, its methyloxime, and 3β-(β,β-dimethylaminoethoxy)-cholest-5-ene inhibited Δ^{24}-sterol reductase, and the first two also inhibited the conversion of sitosterol to fucosterol, the first step in the dealkylation process. Inhibitory effects could be largely reversed by 20-hydroxyecdysone, suggesting that molting hormone biosynthesis was also disrupted by these compounds. Studies with 24,28-iminofucosterol by Fujimoto *et al.* showed that the compound inhibited dealkylation of sitosterol, but not by blocking Δ^{24}-sterol reductase (*21*). The fact that using cholesterol as the dietary sterol did not eliminate the inhibition of development by this compound, indicated involvement of yet another site of action. The steroidal allenes stigmasta-5,24(28),28-trien-3β-ol and cholesta-5,23,24-trien-3β-ol proved to be specific inhibitors of the conversion of sitosterol to fucosterol or of fucosterol to the epoxide when fed at the same concentration as the dietary sterol in studies by Awata *et al.* (*22*). Clarke *et al.* found that the cleavage of fucosterol-24(28)-epoxide to form desmosterol and cholesterol in an *in vitro* preparation from *Spodoptera litteralis* larval midgut was markedly inhibited by the fungicide triadimefon and several related fungicides (*23*). However, only very high concentrations (1000 ppm) of triadimefon were toxic to larvae when fed in the diet. The effects of a series of 29-fluorophytosterols in *Manduca* were reported by Prestwich *et al.* (*24*). Dealkylation of these sterols produces a lethal product fluoroacetate and the inhibitor has no direct effect on sterol metabolism. This novel approach to disrupting insect development utilizes the ability of insects to dealkylate. Since vertebrates are unable to dealkylate, fluorinated C_{29}-phytosterols should have no effect on vertebrates. Of the series of 29-fluorophytosterols tested, 29-fluorostigmasterol was the most active. The Δ^{22}-bond greatly increased the toxicity. A series of fluorinated cholesterols was also tested for activity when fed to *Manduca* and found to have very little toxic effect (*25*). Burger *et al.* found that a number of acetylenic cholesterol derivatives selectively block the C-22 hydroxylase system involved in ecdysone biosynthesis in *in vitro* studies with prothoracic glands and follicular cells from *Locusta migratoria* (*26,27*). The acetylenic function was located at C-22 and the inhibition is specific for the C-22 hydroxylase, is dose dependent and irreversible. This group also examined the effects of a series of allenic cholesterol derivatives synthesized from pregnenolone on ecdysone biosynthesis by prothoracic glands of *Locusta* (*28,29*). These included an allenic function at C-20 or C-22 and were designed to inhibit the C-22 hydroxylation step in ecdysone biosynthesis. Among these compounds a C_{24} compound with a C-22 allenic function was the most efficient inhibitor of this hydroxylation step.

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Inhibition of Ecdysteroid Metabolism. The natural product azadirachtin, a tetranortriterpenoid, in a number of studies has been found to delay or suppress ecdysteroid levels, but little is known of possible direct effects on ecdysteroid biosynthesis or metabolism. Recently, Smith and Mitchell found that azadirachtin inhibited ecdysone 20-monooxygenase in *in vitro* systems prepared from *Drosophila melanogaster*, *Aedes aegypti*, and *M. sexta* in a dose-response fashion (*30*). Koolman *et al.* reported inhibition of this same enzyme by azadirachtin in *in vitro* studies with *Calliphora vicina* (*31*). Pener *et al.* concluded that azadirachtin does not affect prothoracic gland function but post-prothoracic gland ecdysteroid metabolism might be affected (*32*). *In vivo* studies with *Tenebrio molitor* pupae injected with azadirachtin also indicate that the 20-monooxygenase system is inhibited by azadirachtin since 20-hydroxyecdysone levels are much more reduced than are the levels of ecdysone in treated pupae (*33*). The fungicide fenarimol, a known cytochrome P-450 inhibitor, has been shown to directly affect 20-monooxygenase in *in vitro* systems from *Calliphora vicina* and *Neobellieria bullata* (*34*). It had no influence on the biosynthesis of ecdysone. Plumbagin, a chitin synthetase inhibitor, and its 2-demethyl derivative juglone, were reported to inhibit ecdysone 20-monooxygenase activity in *in vitro* systems from *A. aegypti*, *D. melanogaster*, and *M. sexta* (*35*). In related studies with *Dysdercus cingulatus*, plumbagin inhibited ecdysteroid biosynthesis by prothocacic glands *in vitro* and it was concluded that the compound acted directly on the prothoracic glands and that sterol oxidases other than 20-monooxygenase may also be blocked (*36*). The imidazole compound KK-42 has been studied extensively in a number of insect species and shown to have anti-ecdysteroid properties as an insect growth regulator. This interesting compound inhibits ecdysteroid bioysnthesis by prothoracic glands in a non-specific manner in glands from *L. migratoria* (*37*) and *Bombyx mori* (*38*) larvae. Another study of interest, although not involving direct inhibition of steroid metabolism, reported that ethoxyprecocene II appears to act directly on *Rhodnius prolixus* prothoracic glands *in vitro* to interfere with ecdysteroid production (*39*).

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The authors stated that the effects are probably due to destructive alkylation of the glandular tissue.

Other Approaches of Interest. Although space does not allow detailed discussion of these studies, a number of other indirect approaches to disrupting steroid metabolism in insects are being examined in several laboratories. One of the most important of these was the discovery of the extremely active nonsteroidal ecdysone agonist RH-5849 and related compounds by Wing *et al.* (*40*). The effects of altered or unusual dietary phytosterols on insect development (*41*) and ecdysteroid biosynthesis (*42*) indicate another interesting area of research that should be pursued. An indication of the possibilities available through molecular biology techniques is the report of a gene encoding an ecdysteroid UDP-glucosyl transferase enzyme in a baculovirus strain that interferes with molting of the host when injected into *Spodoptera frugiperda* larvae (*43*).

Inhibition of Nematode Sterol Biosynthesis

Nematode Sterol Biochemistry. Parasitic nematodes significantly impact the health and productivity of humans, livestock, and agricultural crops. Despite the multibillion dollar annual loss in agricultural productivity induced by plant-parasitic nematodes, their control has proven difficult. Agricultural nematicides include two classes—soil fumigants and nonfumigant acetylcholinesterase inhibitors. Of the few broad-spectrum agricultural nematicides, several have been withdrawn from use, deregistered or face potential restrictions because of environmental problems or adverse effects on human health. Few viable nematicides remain; there is an urgent need to develop alternative strategies for nematode control. Because sterol biochemistry is one of the few major areas of difference between parasitic nematodes and their hosts, nematode sterol metabolism is a rationally selected target for disruption of important life processes involving steroids.

Knowledge of nematode sterol biochemistry has been obtained more slowly than that of insects because of the limited number of researchers in this area. Like insects, the lack of *de novo* sterol biosynthesis in nematodes results in a nutritional requirement for sterol in the nematode diet or host (*44,45*). It is this dependency upon host sterols that has provided impetus to studies of nematode sterol biochemistry. A detailed presentation of nematode sterol biochemistry is constrained by space but has been published (*2,4*; Chitwood, D. J.; Lusby, W. R.; *Lipids*, in press). Mammalian parasites typically contain the same predominant sterol as their hosts (i.e., cholesterol), except for some gut parasites that contain phytosterols and saturated phytosterols obtained from the host gut contents (*46-49*). One might expect phytoparasitic nematodes to dealkylate plant sterols at C-24 similarly to phytophagous insects; in fact, several species of plant-parasitic nematodes are capable of phytosterol dealkylation (*50-53*). However, a few plant-parasitic species also saturate the phytosterol nucleus (*52-55*); in one such species, the corn root lesion nematode *Pratylenchus agilis*, saturation of the sterol nucleus is the major metabolic transformation of host sterols (*55*). A remaining group of nematodes consists of free-living or microbotrophic nematodes, which feed upon bacteria and other microorganisms in nature, but, unlike parasitic nematodes, may be easily propagated in the laboratory in sterile, host-free medium. These nematodes have been utilized extensively as model organisms to investigate the metabolism of plant sterols by phytoparasitic nematodes. These studies with free-living species such as *Caenorhabditis elegans* unequivocally demonstrated in nematodes the existence of C-24 dealkylation and other sterol metabolic pathways, e.g., hydrogenation of Δ^5-, Δ^{22}-, and Δ^{24}-bonds, introduction of Δ^7-, $\Delta^{9(11)}$-, Δ^{23}-, and $\Delta^{24(28)}$- bonds, $\Delta^7 \rightarrow \Delta^{8(14)}$ isomerization, and C-3 esterification (*56-66*). Most interestingly, a 4α-methylation pathway unique to nematodes was discovered (*58*). More appropriate to the current topic, free-living nematodes have been useful organisms for determining specific effects of sterol biosynthesis inhibitors upon specific metabolic transformations in nematodes.

Biological Effects of Sterol Biosynthesis Inhibitors. The lack of *de novo* sterol biosynthesis in nematodes precludes inhibition of early steps of the sterol biosynthetic pathway. As with insects, the first investigations of sterol metabolism inhibitors in nematodes were performed with compounds with known hypocholesterolemic activity in mammals, e.g., triparanol succinate (*56*). This compound at 40 µg/ml had no biological effect on *Turbatrix aceti* (the vinegar eelworm) but the resultant accumulation of desmosterol in treated nematodes demonstrated the feasibility of altering nematode sterol metabolism. As with insects, azasteroids and long-chain alkylamines and alkylamides have been the most frequently used experimental inhibitors of nematode steroid metabolism.

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Azasteroids. 25-Azacoprostane hydrochloride at 5 µg/ml strongly inhibited growth, development, and motility of *C. elegans* (*59*). In the rat and mouse parasites *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus* (=*Nematospiroides dubius*), 25-azacoprostane or 25-azacholestane at 10 µg/ml retarded development of first-stage juveniles to third-stage juveniles (*67*). The addition of cholesterol to the culture medium reversed the effects of the azasteroids, and azasteroid-treated juveniles and juveniles cultured in sterol-deficient medium displayed similar morphological abnormalities. At 25 µg/ml, 25-azacoprostane decreased adult length and egg production by the free-living species *Caenorhabditis briggsae* and *Panagrellus redivivus* (*68*). Addition of 5 µg/ml cholesterol to culture medium prevented the azasteroid-induced effects in *P. redivivus* but not *C. briggsae*. At 5 µg/ml, 25-azacoprostane and 25-azacholestane inhibited microfilarial production by *Brugia pahangi*; in addition, 25-azacoprostane was macrofilaricidal (*69*).

Amines and Amides. Each of 21 long chain amines and amides examined for activity against *P. redivivus* were directly toxic to this nematode, often at concentrations as low as 5 µg/ml (*70*). Four of the five were also active against the root-knot nematode *Meloidogyne incognita* in greenhouse bioassays with tomato plants. Seven alkylamines inhibited development or killed the medium stomach worm of cattle, *Ostertagia ostertagi*, at 1.0-2.5 µg/ml; sublethal concentrations inhibited molting, decreased motility, induced paralysis, inhibited development to the next life cycle stage, or decreased egg production (*71*). Because the anthelmintic thiabendazole at sublethal concentrations did not have such developmental effects, it was concluded that these compounds might have a hormonal or antihormonal mode of action. Three straight-chain dimethyl alkylamines or an alkylamide analog at 25 µg/ml inhibited reproduction and motility of *C. elegans*; a dimethylalkyl phosphonate ester and a dimethylalkylamide were less active (*62*). *N,N*-Dimethyldodecanamide or *N*-ethyldodecanamine at 10 µg/ml inhibited embryogenesis in eggs of *N. brasiliensis* and *H. polygyrus* (*72*). Four amines (*N*-ethyltetradecanamine, *N*-ethyldodecanamine, and the two *N,N*-dimethyl analogues) at 0.5-5.0 µg/ml strongly inhibited microfilarial production by *B. pahangi* and were macrofilaricidal at 1.0 µg/ml (*69*). Finally, among several dozen amines examined for toxicity against the pinewood nematode, *Bursaphelenchus xylophilus*, some (e.g., octadecylamine and *N,N*-dimethyloleylamine) were active at concentrations as low as 2 µg/ml (*73,74*).

Other Inhibitors. There have been few investigations of biological activity of additional classes of sterol metabolism inhibitors on nematodes. Cholestyramine resin (5.0%) or clofibrate (0.1%) inhibited growth and development of the insect-parasitic nematode *Steinernema feltiae* (=*Neoaplectana carpocapsae*) (*75*). At 50 µg/ml, 29-fluorostigmasterol, a compound that is toxic to insects because of liberation of fluoroacetate during C-24 dealkylation (*24*), inhibited reproduction of *C. elegans* (*76*). Various fungal C-14 demethylase inhibitors—miconazole, fenarimol, ketoconazole, triarimol, or triadimefon—had no visible effects on *C. elegans* when incorporated into standard sitosterol-containing growth medium (*64*) at 50 µg/ml (Chitwood, unpublished data). The biological effects of ecdysteroids and vertebrate steroid hormones upon nematodes are beyond the scope of this review but have been summarized recently (*2,77*).

Biochemical Effects of Sterol Biosynthesis Inhibitors. The accumulation of desmosterol in *T. aceti* treated with triparanol succinate (*56*) indicated that sterol metabolism in nematodes and insects could be similar, as desmosterol also accumulates in insects treated with Δ^{24}-sterol reductase inhibitors (described above). Several years later, discovery of the nematotoxic activity of azasteroids and long-chain alkylamines and alkylamides stimulated investigation of their mode of action. When 5 µg/ml 25-azacoprostane hydrochloride was incorporated into sterile culture medium containing sitosterol, over 96% of the sterols from *C. elegans* (excluding the dietary sitosterol) were Δ^{24}- or $\Delta^{24(28)}$-sterols, such as fucosterol, cholesta-5,7,24-trienol, desmosterol, and cholesta-7,24-dienol (*59*). These sterols were detectable in no more than trace quantities in control nematodes. (Unlike most insects and some other nematodes, *C. elegans* usually contains a large amount of 7-dehydrocholesterol; hence the abundance of sterols with a Δ^7-bond.) Thus, the azasteroid clearly inhibited the *C. elegans* Δ^{24}-sterol reductase enzyme system. This inhibition and the previously described reversal of many of the biological effects of azasteroids upon nematodes by addition of cholesterol to the culture system indicates that the biological activity of azasteroids in nematodes largely results from inhibition of the Δ^{24}-sterol reductase of nematodes. Of course, additional loci of inhibition may exist; indeed, 25-azacoprostane also inhibited a $\Delta^7 \rightarrow \Delta^{8(14)}$-sterol isomerase, as most of the 4α-methylsterols in treated nematodes had a Δ^7-bond

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instead of the $\Delta^{8(14)}$-bond that normally occurs in the 4α-methylsterols of this species (*58*).

Several aliphatic amines (*N*,*N*-dimethyldodecanamine, *N*,*N*-dimethyltetradecanamine, *N*,*N*-dimethylhexadecanamine, and *N*,*N*-dimethyl-3,7,11-trimethyldodecanamine), an amide (*N*,*N*-dimethyldodecanamide), and a phosphonate ester (dimethyl 1-dodecanephosphonate) were also examined for biochemical effects on *C. elegans* propagated in sitosterol-containing medium (*62*). All four amines inhibited the Δ^{24}-sterol reductase of *C. elegans*, whereas the amide and phosphonate ester did not. The branched-chain amine inhibited the Δ^{24}-sterol reductase to the greatest extent; inhibitory activity of the other amines was inversely proportional to chain length. Neither the amide nor the phosphonate ester inhibited the reductase. Interestingly, *N*,*N*-dimethyl-3,7,11-trimethyldodecanamine also blocked the conversion of sitosterol to fucosterol (one of the initial steps in C-24 dealkylation), possibly because of the resemblance of the amine to the side chain of sitosterol. The lack of correlation of biological activity of the amines with their inhibition of Δ^{24}-sterol reductase indicates that their mode of action is not via inhibition of this enzyme system. Alteration of other steroid metabolic pathways (as in insects) remains a possibility, although the immediate disruption of movement upon transfer of nematodes to amine-containing medium would argue against this explanation.

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Although not biologically active against *C. elegans* because of its effects on phytosterol dealkylation, *N*,*N*-dimethyldodecanamine has been used as a metabolic probe to elucidate pathways involved in the metabolism of campesterol and stigmasterol by nematodes (*63*). These investigations led to the discovery of many previously unidentified metabolic intermediates in nematodes and confirmed that stigmasterol and campesterol dealkylation involves a Δ^{24}-sterol reductase, with 24-methylenecholesterol and stigmasta-5,22,24(28)-trienol being key intermediates, respectively. Reduction of the Δ^{24}-bond in the latter intermediate occurred only after reduction of the Δ^{22}-bond. Amine-treated nematodes propagated in campesterol-supplemented medium accumulated unmetabolized dietary campesterol.

The biochemical effects of few other potential inhibitors of nematode sterol metabolism have been characterized. At 50 μg/ml in sitosterol-supplemented medium, AY-9944 (*trans*-1,4-bis[2-chlorobenzylaminomethyl]cyclohexane dihydrochloride), a Δ^7-isomerase inhibitor (*78-80*), inhibited the $\Delta^7 \rightarrow \Delta^{8(14)}$-sterol isomerase in *C. elegans* (*76*). Given the dependency of parasitic nematodes upon their hosts for sterols, the large variety of metabolic transformations performed upon sterols by nematodes, and the uniqueness of one of these (i.e., 4α-methylation), further exploration of this potentially exploitable area of nematode biochemistry is imperative.

Conclusions

Much basic information on sterol metabolism and utilization in insects and nematodes has been developed and many parallels between these two groups of organisms have become obvious. This is particularly true with respect to the dealkylation and conversion of C_{28} and C_{29} phytosterols to cholesterol where the pathways appear to be very similar (Figure 1). The major difference between

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insects and nematodes with regard to steroid biochemistry to date, is that the biosynthesis of ecdysteroids from cholesterol or other precursors has not been demonstrated in nematodes. The similarities in the pathways of neutral sterol metabolism in insects and nematodes is further emphasized by the similar effects of Δ^{24}-sterol reductase inhibitors such as azasteroids, alkylamines and alkylamides in both groups of organisms. This demonstrates that sterol metabolism in both insects and nematodes could be specifically inhibited. Other steps in the dealkylation of the sterol side chain are susceptible to inhibition by such compounds as iminosteroids, allenes, and certain fungicides. Fluorinated sterols take advantage of differences in steroid biochemistry between insects and nematodes and other organisms. Side chain hydroxylation is also a likely target in ecdysteroid biosynthesis in insects as shown by the inhibition of C-22 hydroxylation by certain acetylenic and allenic cholesterol derivatives. Ecdysone 20-monooxygenase is inhibited by azadirachtin and fungicides such as fenarimol as well as the natural product plumbagin. Other inhibitors have shown less specific effects on steroid metabolism. The research discussed in this brief review provides very promising leads in the search for practical means of exploiting this important area of biochemical difference in the development of new, selective methods of pest control.

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Chapter 16

Cytochrome P450-Dependent 14α-Demethylase

Target for Antifungal Agents and Herbicides

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The imidazole derivatives, followed by the triazoles, signalled a new era of broad-spectrum, safe, topically and orally effective antifungal agents. All belong to the class of 14α-demethylase inhibitors (*1-2*). Their antifungal activity originates from binding to a cytochrome P450 ($P450_{14DM}$) involved in the 14α-demethylation of lanosterol (in *Saccharomyces cerevisiae* and *Candida glabrata*) or 24-methylenedihydrolanosterol (in filamentous fungi, *Cryptococcus neoformans*, the yeast form of *Histoplasma capsulatum* and a number of *Candida albicans* isolates).

Cytochromes P450 (P450) are present in just about every phyla in which they have been sought. The prokaryote forms are soluble proteins, whereas in eukaryotes they are membrane-bound (*e.g.* mitochondrial inner membrane and endoplasmic reticulum). P450s catalyze the synthesis and/or metabolism of a long list of key-compounds. Examples are sterols (*e.g.* ergosterol, cholesterol and phytosterols such as campesterol, sitosterol and stigmasterol), steroids (*e.g.* androgens, cortisol and estrogens), bile acids, thromboxane A2, prostacyclin, leukotrienes (*3*). They also play an important role in the activation of vitamin D, the metabolism of retinoic acid and xenobiotics (*3*). Most of the P450s are classified as monooxygenases. They catalyze the following reaction:

$$\mathrm{NADPH{+}\ H^+ + O_2 + SH \Rightarrow NADP^+ + H_2O + SOH}$$

In this reaction SH represents a substrate (for example 24-methylenedihydrolanosterol) to be oxidized by the P450 enzyme. The reaction requires molecular oxygen of which one oxygen atom is inserted into the substrate while the other oxygen atom is reduced to water. $NADPH_2$ provides the electrons needed for the activation of this process. The active site of P450 contains a ferric prostetic heme group, the substrate binds to the protein moiety of the P450 and the heme iron is reduced . The specific enzymatic function of the P450s, i.e. to activate oxygen for insertion into a substrate, originates from the electronic structure of the heme iron linked to the thiol of a cysteyl residue of

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the P450 protein moiety. Carbon monoxide competes with oxygen for the same binding place i.e. the reduced heme iron. From the specific interaction of the heme group with the protein originates the typical absorption maximum at 450 nm of the reduced carbon monoxide-P450 complex (the name cytochrome P450 describes a reduced- carbon monoxide binding pigment which absorbs at about 450 nm).

Effects of Azole Antifungals on Fungal Cytochromes P450

Azole antifungals, such as miconazole, imazalil, ketoconazole, itraconazole, terconazole, saperconazole and fluconazole (chemical structures are given in Figure 1), bind to the heme iron via their azole ring (imidazole N-3 or triazole N-4) and to the protein moiety of the P450 via the substituent on N-1 of this ring (*1*, *4-7*). They compete in this way with oxygen and CO binding. For example fifty % inhibition of CO-binding to microsomal P450 from *C. albicans* (8) or *C. glabrata* (Vanden Bossche *et al.*, *Am. J. Obst. Gyn.*, in press.) is obtained with nanomolar concentrations of the imidazole derivatives, miconazole and ketoconazole, and the triazoles terconazole, itraconazole and saperconazole (*8*). Another triazole derivative, fluconazole, is a 8-times less potent inhibitor of CO-binding (*8*). This already indicates that the potency of an azole derivative is not only determined by its binding to the heme iron. It has been shown previously that itraconazole and its imidazole analogue (R 49960) form equistable complexes with *C. albicans* P450, the less hydrophobic ketoconazole forms a less stable P450 complex and the stability of the complex is not increased by replacing the imidazole ring by a triazole (R42164) (*6*). Thus, although the binding to the heme iron is a prerequisite for inhibition, the binding of the N-1 substituent to the protein moiety determines the potency.

Comparing the effects of ketoconazole with those of nor-ketoconazole (deacyl ketoconazole) indicates that minor structural changes in the N-1 substituent affect the interaction with P450. Nor-ketoconazole has 2 and 3 times lower affinity for the microsomal P450 from piglet testis and *C. albicans*, respectively (*9*). Furthermore, the triazole derivative R 76713 (Figure 2) and its (+)enantiomer, R 83842 are potent and highly selective inhibitors of CO-binding to the human placental microsomal P450 (*10*) but don't have any effect on the microsomal P450s of *C. albicans*. These results suggest that the *N*-1 substituent (non-ligand hydrophobic part) of the azoles also has a greater impact on the selective interaction with P450 enzymes than the nature of the nitrogen heterocycle.

Effects of Azole Antifungals on Ergosterol Biosynthesis

A major P450 present in *C. albicans* microsomes is involved in the 14α-demethylation of lanosterol or 24-methylenedihydrolanosterol i.e. $P450_{14DM}$. Thus, the effects of azole antifungals on microsomal P450(s) of *C. albicans* are suggestive for an interaction with the 14α-demethylase and hence with ergosterol synthesis. Inhibition of ergosterol biosynthesis has been shown with *e.g.* imazalil, miconazole, terconazole, ketoconazole, itraconazole and/or saperconazole in *C. albicans*, *C. glabrata*, *C. lusitaniae*, *Pityrosporum ovale*, *Trichophyton mentagrophytes*, *Paracoccidioides brasiliensis*, *Histoplasma capsulatum* , *Cryptococcus neoformans*, *Aspergillus fumigatus* and/or *Penicillium italicum* (*1-9*, *11-15*, Vanden Bossche, H., unpublished data). For example, complete ergosterol depletion is achieved when *C. albicans* is incubated with 10-30 nM itraconazole (*9*). It should be noted that ergosterol depletion is a prerequisite to block cell proliferation (*16*). A summary of the concentrations of itraconazole and fluconazole needed to reach 50% inhibition of ergosterol synthesis in a number of pathogenic fungi is given in Table I. The higher concentrations of fluconazole needed to inhibit ergosterol synthesis (Table I) originate, at least partly, from its lower effect on carbon monoxide binding to fungal P450s. However, since 140- 1600-times more fluconazole is needed to achieve 50% inhibition of ergosterol synthesis in intact cells of

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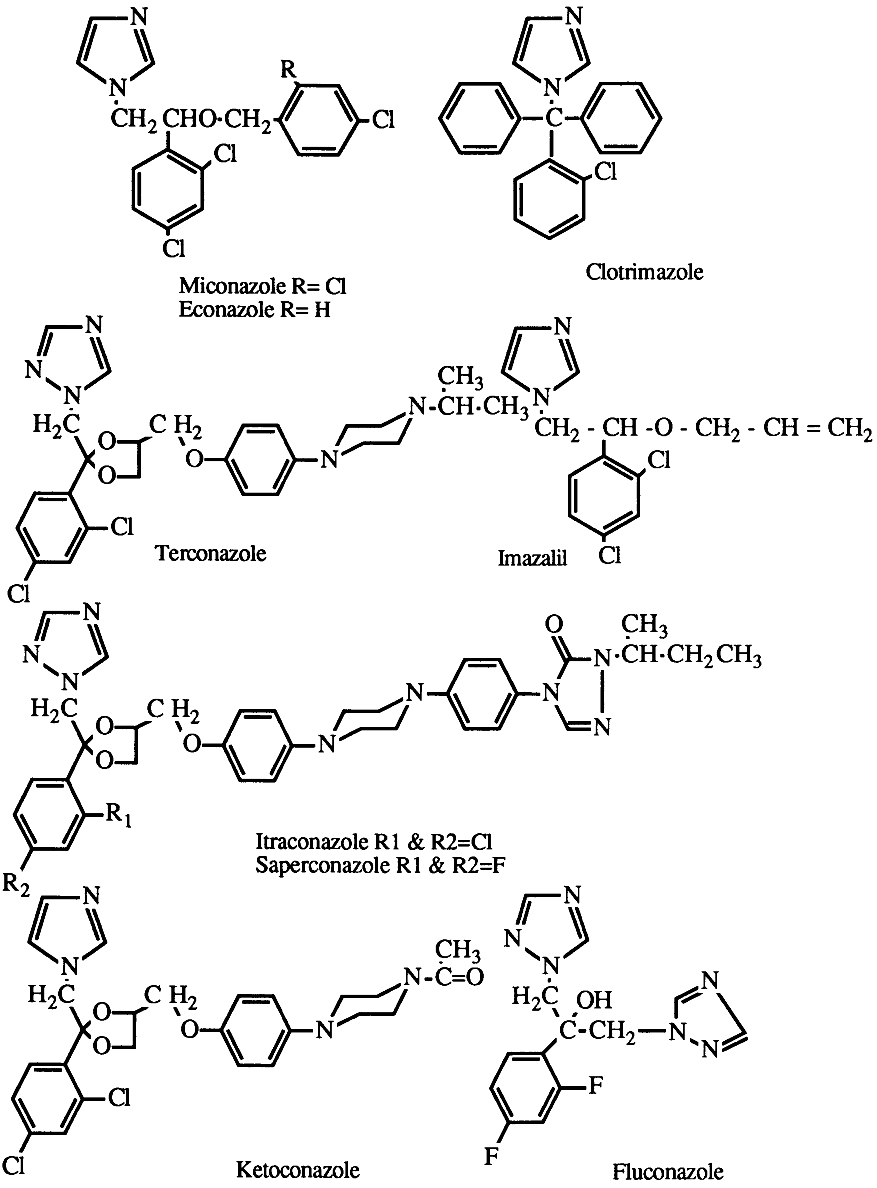
Miconazole R= Cl
Econazole R= H
Clotrimazole
Terconazole
Imazalil
Itraconazole R1 & R2=Cl
Saperconazole R1 & R2=F
Ketoconazole
Fluconazole

Figure 1- Chemical structures of azole antifungals

C. albicans or *C. glabrata* and about 8-times more to get 50% inhibition of CO-binding to the P450s (*supra vide*) other factors are involved *e.g.* only a small portion of the added fluconazole may reach the target enzyme i.e. the P450-dependent 14α-demethylase.

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Inhibition of ergosterol synthesis coincides with the accumulation of 14-methylated sterols. This proves that inhibition of ergosterol synthesis originates from interaction with the P450-dependent 14α-demethylase. For example, when *C. albicans* is incubated with nanomolar concentrations of itraconazole radioactivity derived from [^{14}C]-acetate is found in 14α-methylfecosterol, obtusifoliol, 24-methylenedihydrolanosterol and especially 14α-methyl-ergosta-8,24(28)-dien-3β,6α-diol (3,6-diol) (*9*) (Table II).

The 6-hydroxylation may be part of the $\Delta^{5,6}$ double bond insertion, one of the last reactions in ergosterol synthesis. The accumulation of this 3,6-diol in azole antifungal

Table I. Effects of Itraconazole and Fluconazole on Ergosterol Synthesis

Species	Growth Conditions[a]	IC$_{50}$-Values (nM)[b]	
		Itraconazole	Fluconazole
C. albicans (ATCC 28516)	16h, 37°C, CYG	4.8	670
C. glabrata (B 16205)	16h, 37°C, CYG	24	40,000
Cr. neoformans[c] (B 42419)	16h, 37°C, PYG	11	8,300
H. capsulatum[c] (G217B)	48h, 37°C, GYC	3	21,000

[a] CYG = Casein hydrolysate - Yeast extract - Glucose (each 5 g/l). PYG = Polypeptone - Yeast extract - Glucose (each 1 g/l). GYC = Glucose (2 %) - Yeast extract (1%) - Cysteine (0.03 %).

[b] IC$_{50}$-Value = concentration needed to get 50 % inhibtion of ergosterol synthesis from [^{14}C]-acetate.

[c] Under control conditions *Cr. neoformans* forms next to ergosterol (83 %) 5-dihydroergosterol (2 %), 22-dihydroergosterol (6 %) and fungisterol (ergost-7-enol; 9 %). *H. capsulatum* forms next to ergosterol (63 %) also brassicasterol (22 %) and 22-dihydroergosterol (15 %).

Table II. Sterols accumulating in the presence of Itraconazole[a]

Sterols	% of Total Radioactivity			
	C. albicans (30 nM)	*C. glabrata* (3 μM)	*Cr. neoformans* (30 nM)	*H. capsulatum* (10 nM)
14α-Methyl-ergosta-$\Delta^{8,24(28)}$-dien-3β,6α-diol	71	53	4	4
14α-Methyl-ergosta-$\Delta^{5,7,22,24(28)}$-tetraen	1	1	0	0
14α-Methyl-fecosterol	7	14	0	0
Obtusifoliol	8	1	5	2
14α-Methyl-fecosterone	0	0	1	6
Obtusifolione	0	0	30	32
Lanosterol	8	12	0	2
Eburicol	4	0	30	38

[a] Incubations, homogenisation, extraction of sterols, separation by HPLC and identification were as described previously (*2*, *9*, *14*). Figures in parenthesis represent the concentrations of itraconazole used. Eburicol= 24-methylenedihydrolanosterol.

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treated *Candida* may originate from the inability of the $\Delta^{5,6}$ -desaturase to use 14-methylsterols as substrates. 14-Methylated sterols accumulating in *C. glabrata* and *H. capsulatum* are also given. In contrast with *C. albicans*, *C. glabrata*, incubated in the presence of itraconazole, does not accumulate eburicol. This suggests that, as in *Saccharomyces cerevisiae*, lanosterol may be the substrate for the 14α-demethylase. A block in ergosterol synthesis coincides in *H. capsulatum* with the accumulation of 14-methylated sterols and the 14-methylated ketosteroids obtusifolione and 14-methylfecosterone. Similar results are obtained with ketoconazole (*2*). *Cr. neoformans* incubated in the presence of increasing concentrations (5 nM- 10 μM) of itraconazole also accumulates obtusifolione (Vanden Bossche, H., unpublished data). Both ketosteroids are intermediates formed during the 4-demethylation of eburicol and obtusifoliol. The accumulation of these ketosteroids suggests that itraconazole and ketoconazole inhibit, next to the 14α-demethylase also the 3-ketoreductase. Studies are on-going to determine whether the 3-ketoreductase in both *Cr. neoformans* and *H. capsulatum* is a target for ketoconazole and itraconazole or that the observed inhibition is secondary to their interaction with the 14α-demethylase.

Conclusive evidence has been collected showing that the 14-methylated sterols induce permeability changes, membrane leakiness, changes in membrane-bound enzymes, inhibition of growth and cell death (*1*, *16-17*). For example one striking change is the increased and uncoordinated synthesis of chitin. Patches of chitin are found over the cell wall instead of being localized at the septa and growth tip (*13*, *17*). This accumulation of chitin disturbs the normal sequence of cell separation, resulting in clusters of interconnected cells and causing abnormal swelling and bursting of cells (*13*, *17*). Other membrane-bound enzymes are also affected (for a review see Ref. 17). One example: the nature of the acyl moieties of the phospholipids of *C. albicans* changes after 16 h of growth in the presence of 10 nM ketoconazole or miconazole. Under control conditions oleic acid (18:1) is the major fatty acid present, whereas in the presence of the azole antifungals palmitic acid (16:0) is the predominant fatty acid (*17-18*). An increase in palmitate has also been found by Georgopapadakou *et al.* (*19*) in *C. albicans* grown in the presence of 1 μM clotrimazole, econazole, miconazole or ketoconazole. The shift from unsaturated to saturated fatty acids suggests an inhibition of the NADH-dependent fatty acid desaturase, an enzyme that requires the presence of a microsomal electron transport system that consists of NADH cytochrome b5 reductase and cytochrome b5. All three components of the system are embedded in the microsomal membranes and are affected by the fluidity of the membrane (*20*).

Effects of Azole Antifungals on mammalian P450s

A great number of cytochromes P450 are involved in the synthesis and metabolism of endobiotics in mammalian cells. Therefore, topically and orally active antifungals whose activity is based on the inhibition of a P450-dependent process should be evaluated for possible interactions with mammalian P450 enzymes.

It is obvious that the first candidate to be tested is $P450_{14DM}$ involved in cholesterol synthesis. Fifty % inhibition of cholesterol synthesis from mevalonate in subcellular fractions of male rat liver is reached at 2 μM ketoconazole and 6 μM miconazole (*21*). To obtain 50% inhibition of the 14α-demethylation of lanosterol in phytohemagglutinin (PHA)- stimulated human peripheral lymphocytes, 0.1 μM of ketoconazole is needed (*22*). When macrophages (*23*) or human hepatoma cells (Hep G2 cells) (Vanden Bossche, H., unpublished data) were incubated in the presence of [^{14}C]-acetate and ketoconazole, 50% inhibition of cholesterol synthesis was achieved at 1.5 μM and 1.2 μM, respectively. To reach 50% inhibition in Hep G2 cells 1.3 μM of itraconazole is needed (Table III). Fluoro substituents on the phenyl ring (saperconazole, Figure 1) provide a lower activity than the corresponding chloro substituents (itraconazole). Indeed, even at 10 μM of saperconazole an inhibition of only 36 % is found (*14*).

All these studies indicate that miconazole, ketoconazole, itraconazole and saperconazole inhibit cholesterol synthesis at concentrations higher than those needed to inhibit lanosterol 14α-demethylase in *Candida*.

At concentrations 10-times lower than those needed to inhibit cholesterol synthesis, ketoconazole inhibits the conversion of 17α-hydroxy,20-dihydroprogesterone into androstenedione by rat testicular microsomes; 50% inhibition is achieved at 0.2 μM (*24-26*). These studies prove that ketoconazole is an inhibitor of the P450-dependent 17,20-lyase. Ketoconazole also interferes with adrenal androgen synthesis (*27-28*). The effects on adrenal and testicular androgen synthesis have made ketoconazole a good candidate for the treatment of androgen dependent prostate carcinoma (*27, 29-30*). At the high dose (400 mg every 8 hours instead of 200 mg daily in the treatment of mycoses) used, the principal side effect is gastric discomfort with nausea (*31*) which resolves with administration of antinauseants or upon discontinuation of the drug (*32*). Thus, ketoconazole, at high doses, is effective both clinically and endocrinologically in the treatment of metastatic prostate carcinoma, but its use is limited by gastric discomfort. However, these investigations provided us with a battery of P450 systems to evaluate activity and predict possible toxicity and have triggered a multidisciplinary study to open new possibilities in medical treatment. For example, the study of these P450s was of help in the development of itraconazole, which is almost devoid of effects on mammalian P450-dependent reactions (Table III) and of R 83842 (see above) which is a selective inhibitor of the P450-dependent aromatase.

Table III. Effects of Itraconazole on P450-dependent Reactions in Intact Cells and Microsomal or Mitochondrial Membranes[a]

Species	Organ Cells	Membranes	Substrate	Product formed	IC50 (μM)
Rat	Liver	S-10,000	Mevalonate	Cholesterol	7
		Microsomes	Cholesterol	7-OH-cholesterol	>10
		Microsomes	Testosterone	Polar metabolites	>10
	Testes	S-10,000	Pregnenolone	Androgens	>5[b]
	Skin	Microsomes	Retinoic acid	4-OH-retinoic acid	>10
Bovine	Adrenals	Mitochondria	Cholesterol	Pregnenolone	>5[b]
		Mitochondria	11-Deoxy-cortisol	Cortisol	>5[b]
		Microsomes	Pregnenolone	17-OH-pregnenolone	>5[b]
		Microsomes	Pregnenolone	DOC	>10
		Microsomes	Pregnenolone	Androgens	>5[b]
Human	Platelets	Microsomes	Arachidonic acid	Thromboxane	>10
	Liver	Chang cells	Acetate	Cholesterol	0.76
		Hep G2 cells	Acetate	Cholesterol	2.8
	Placenta	Microsomes	Androstene-dione	Estrogens	>10
	Testes	Microsomes	Pregnenolone	Androgens	>10

[a] S-10,000= the supernatant of a 10,000 *g* centrifugation. [b] Limit of solubility. Most data are taken from Ref. 2.

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Interactions of Azole derivatives with Plant P450s

High concentrations of the azole agricultural antifungals, imazalil (Figure 1) and propiconazole (Figure 2) are needed to inhibit carbon monoxide binding to microsomal P450s from Jerusalem artichoke tubers, pea and maize seedlings (5). This indicates that both azoles show some, albeit low, affinity for plant P450s. The low affinity of imazalil for plant P450s was further proven by the the rather high concentrations (≥ 0.5 μM) needed to obtain a Type II binding spectrum when added to microsomal preparations from Jerusalem artichoke tubers and maize seedlings (5). It should be noted that the microsomes used contain the pool of all P450s. Therefore it is possible that imazalil and propiconazole interact with P450(s) representing a small part only of the total P450 content. In plants, P450s are involved in for example the 14-demethylation of obtusifoliol, the 4-hydroxylation of the monoterpene alkaloid precursor geraniol, in oxidative steps which lead from kaurene to gibberellins and in the 4-hydroxylation of *trans*-cinnamic acid to form *trans*-p-coumaric acid, a precursor of flavonoids and lignin.

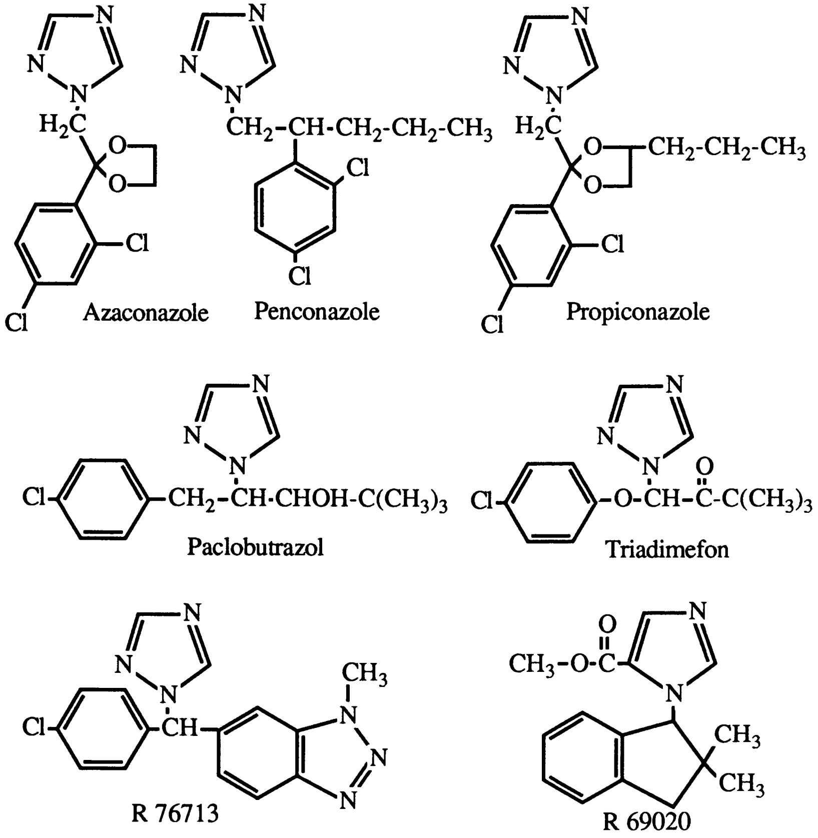

Figure 2- Chemical structures of azole derivatives

At concentrations up to 10 μM, imazalil, azaconazole, penconazole (Figure 2) and propiconazole do not inhibit the cinnamic acid 4-hydroxylase in microsomal fractions of maize shoots (5). Studies of Buchenauer suggest that at 250 μM, imazalil and the other ergosterol biosynthesis inhibitor, triadimefon, have some effect on gibberellin biosynthesis (*33*). However, the 2*S*,3*S*-enantiomer of the triazole paclobutrazol is a potent inhibitor of the three steps in the oxidation of the gibberellin precursor, *ent*-kaurene to *ent*-kaurenoic acid in cell-free extracts of *Cucurbita maxima* endosperm. Fifty % inhibition is obtained at 20 nM (*34*). This enantiomer possesses a high level of plant growth regulatory activity whereas the 2*R*,3*R*-enantiomer has high activity against cereal mildews and rust (*35*). The 2*R*,3*R*-enantiomer is a more potent inhibitor of ergosterol biosynthesis than the 2*S*,3*S*-enantiomer (*36*). Other triazole derivatives such as propiconazole and triadimefon have also been found to inhibit sterol synthesis in maize seedlings with a concomitant accumulation of obtusifoliol, 14-dihydroobtusifoliol and 14α-methyl-24-dihydrofecosterol (37). Both azole antifungals also inhibit the 14α-demethylation of obtusifoliol in microsomes prepared from maize seedlings. Fifty % inhibition is achieved at 2 μM propiconazole and 8 μM triadimefon (37). Both compounds are more potent inhibitors of lanosterol 14α-demethylation in microsomal preparations of yeast cells. For example, Yoshida and Aoyama (*38*) using *S. cerevisiae* microsomes obtained almost 95 % inhibition with 2 μM triadimefon, and over 95 % inhibition of the lanosterol 14α-demethylation in microsomes prepared from *Candida tropicalis* or *Saccharomyces uvarum* is achieved at 0.5 μM propiconazole (*39*).

The plant 14α-demethylase differs from the fungal and mammalian enzyme in using obtusifoliol, 24(28)-dihydro-obtusifoliol, 24(25)-dihydro-31-norlanosterol and 14α-methyl-24(28)-dihydrofecosterol and not lanosterol or 24-methylene-dihydrolanosterol as substrates (*40*). This indicates that in contrast with fungi and animals the 14α-demethylation in plants is processed only after the 4α–demethylation step. The azole antifungals coordinate with the P450-iron and bind to a region of the apoprotein near the heme or substrate binding domain. Differences in sensitivity to azole antifungals and substrate specificity may result from differences in the amino acid sequence in or near these domains. Knowledge of this sequence may be of help in the development of selective inhibitors of plant sterol synthesis.

The herbicide methyl-1-(2,2-dimethylindan-1-yl)-imidazole-5-carboxylate (R 69020/CGA 201-029, Figure 2) inhibits ergosterol synthesis in *C. albicans* and *C. glabrata*. Fifty % inhibition is reached at 1.7 μM and 0.16 μM, respectively. More than 10 μM is needed to get 50 % inhibition of cholesterol synthesis in human hepatoma cells (Hep G2 cells) and human liver cells (Chang cells). However, after 1week of growth of maize seedlings in the presence of 0.1 μM R 69020 changes in the sterol composition are observed (*41*). Stigmasterol, sitosterol and campesterol are virtually absent in roots of 7-day-old maize seedlings. Instead of these 14-demethylsterols, obtusifoliol and 14α-methylergosta-8-en-3ß-ol are accumulating. These 14-methylsterols are already found after 7 days of growth in the presence of 10 nM. As shown in Table IV, R 69020 also changes the sterol pattern in mung bean roots. However, in contrast with the maize seedlings still high amounts of stigmasterol, sitosterol and fucosterol are present in the roots after 7 days of growth in the presence of 1 μM R 69020. The decrease in 14-demethylsterols coincides with the appearance of obtusifoliol, 4,14-dimethylergosta-8,24-dien-3ß-ol, 14-methylstigmasta-8-en-3ß-ol and 14-methylergosta-8-en-3ß-ol. These data suggest that R 69020 is an inhibitor of the plant P450-dependent obtusifoliol 14α-demethylase. The sterol pattern in the leaves was not altered at the concentration of R 69020 used, probably because of limited transport of the inhibitor.

Further evidence for R 69020's effect on the synthesis of phytosterols was obtained by studying the effects of this imidazole carboxylate on sterol synthesis from [14C]-mevalonic acid in tobacco cells. Suspension cell cultures of *Nicotiana tabacum* were grown in Murashige and Skoog medium (MS -medium; Sigma M5519 supplemented with 30 g sucrose, 2 mg 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg kinetin per liter, pH 5.8). Cells were cultivated at 25 °C in an orbital shaker (110 rpm, exposed to Gro-lux lamps, 400-700 nm, for 16 h per day. Cells (450 mg fresh weight) from a seven day old culture were used to inoculate 30 ml of MS medium.

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After 6 days of incubation, increasing concentrations of R69020 and/or solvent (DMSO 0.1 %) and 1.5 µCi ^{14}C mevalonic acid DBED salt were added. After 24 h of growth, cells were collected onto glassfilters, washed with saline and weighed. Cells were suspended into 10 ml of saline and homogenized for one minute with a Polytron® homogenizer. One volume of 15% KOH dissolved in 90% ethanol was added. The mixtures were refluxed for one hour. Sterols were extracted with one volume of heptane, separated by HPLC (LiChroCart 250-4 HPLC cartridge filled with Superspher 60 RP-8, 244 x 4 mm, Merck-Darmstadt) and identification was done by using standards or with GC-MS identified accumulation products.

Table IV. Sterols in Mung Bean Roots grown in the Presence of R 69020 and/or Solvent[a]

Sterols	Content (µg/mg lyophilizate)	
	Control	R69020 (1 µM)
Stigmasterol	7.30	5.22
Sitosterol	4.50	3.00
Fucosterol	1.20	0.58
Obtusifoliol	0.00	1.40
4,14-Dimethylergosta-8,24-dien-3ß-ol	0.00	0.60
14-Methylstigmasta-8-en-3ß-ol	0.00	1.58
14-Methylergosta-8-en-3ß-ol	0.00	0.84

[a] Mung bean seedlings are grown for 7 days (temperature: 24°C during the day, 22°C at night; humidity: 60 %; Light: ±16,000 lux, 16h light, 8h dark) in the presence of R 69020 (150 ml water containing 0.1 % aceton and 1 µM R 69020/day) or 0.1 % aceton in 150 ml water. Roots are collected 24 h after last treatment, washed, minced, frozen in liquid nitrogen, lyophilized and stored at -30°C. 0.5 g lyophilizate is homogenized in 30 ml of chloroform: methanol (2: 1) using a Braun homogenizer (2 x 1 min). After 30 min of shaking the chloroform phase is collected and dried under a stream of nitrogen. The extract is mixed with 2.5 ml of KOH (15 % in 90% ethanol) and 2.5 ml of water and saponified at 85°C under reflux for 2 h. Sterols are extracted with 15 ml n-heptane, separated and identified by HPLC, gas chromatography and mass spectrometry (*5*).

After 24h of growth in the presence of increasing concentrations of R 69020 (0.1 nM up to 10 µM) the synthesis of 14-demethylsterols (e.g. fucosterol, campesterol, stigmasterol, sitosterol) is inhibited with a concomitant accumulation of the 14-methylated sterol, obtusifoliol (Figure 3). Fifty % inhibition of 14-demethylsterol synthesis is achieved at 4.2 nM; 50 % inhibition of sitosterol synthesis is already reached at 2.7 nM. The higher IC_{50}-value obtained when all 14-demethylsterols are taken together may result from the increased radioactivity incorporated into a fraction with the same retention time as stigmasterol. The HPLC profile of this fraction suggests the presence of at least three different sterols, part of them may be 14α-methylsterols. One component of the heptane extract could not be identified so far. However, since its content is decreasing in the presence of R 69020 it may be a 14α-methylsterol. Further studies are ongoing to identify the different sterols present in these fractions.

The accumulation of obtusifoliol instead of cycloartenol further supports the results of Taton and Rahier (*40*) indicating that obtusifoliol is the best substrate for the P450-dependent 14α-demethylase of plants. The results also prove that R 69020 is a more potent inhibitor of the obtusifiol 14α-demethylase than of the analogous enzyme in *C. albicans* and *C. glabrata*.

Conclusive evidence has been collected showing that the antifungal activity of the azoles originates from their inhibitory effects on the fungal P450-dependent 14α-demethylase. Therefore, it is tempting to speculate that the herbicidal activity of R 69020 also results from its effect on sterol synthesis.

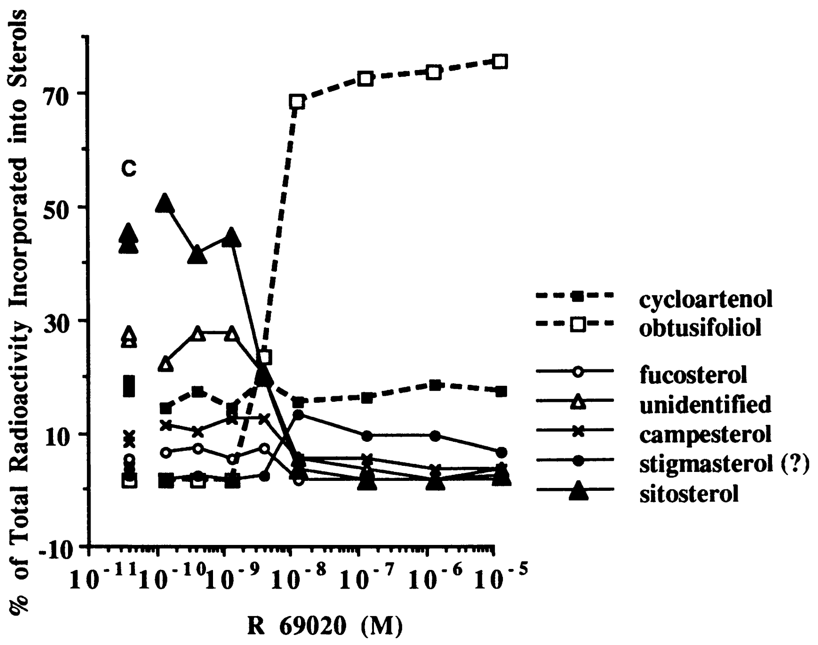

Figure 3- Effects of R 69020 on sterol synthesis in tobacco cells. Results are expressed as % of the sum of the radioactivities found in the different sterols separated by HPLC. The fraction with the same retention time as stigmasterol contains at least three different sterols. The retention time of one fraction (unidentified) did not correspond with those of the available sterol standards.

Conclusion

The results discussed prove that the cytochrome P450-dependent 14α-demethylase is a target enzyme for azole antifungals and for the herbicide methyl-1-(2,2-dimethylindan-1-yl)-imidazole-5-carboxylate (R 69020/CGA 201-029). They also show major differences in the sensitivities of the mammalian, fungal and plant 14α-demethylases for these inhibitors, indicating that P450 enzymes having the same catalytic function are not necessarily identical. Major differences in the affinity of the azole antifungals for mammalian and plant P450s are also described. Furthermore, examples are given which indicate that minor changes in the chemical structure may affect activity and selectivity. Finally, the examples discussed in this overview demonstrate that P450 systems can be exploited in the search for compounds that may become important tools in chemotherapeutic and agrochemical applications.

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Chapter 17

Effect of Tridemorph on Sterol Synthesis in Algae

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Tridemorph was added to the culture medium of *Chlorella sorokiniana* at concentrations of 1 ppm and 2.5 ppm (3.3 and 8.3 x 10^{-6}M), reducing the growth rate by approximately 50% and 75%, respectively. Tridemorph at a concentration of 1 ppm completely inhibited the production of ergosterol, the normal end product of sterol synthesis in this organism. 24-Methylpollinastanol, 24-methylenepollinastanol, and ergost-8(14)-enol accumulated in a 2:3:1 ratio, respectively. At a tridemorph concentration of 2.5 ppm desmethyl sterols were not detected, but 24-methylpollinastanol, 24-methylenepollinastanol, and 24-methylenecycloartanol accumulated in a ratio of 5:4:3, respectively. The results demonstrate that the lack of C-24 ethyl sterols in *Chlorella* is not due to its inability to produce the 24-methylene precursor.

Tridemorph (4-tridecyl-2,6-dimethylmorpholine) is an ergosterol biosynthesis inhibitor in many fungi (1). It has been shown to inhibit the Δ^8 to Δ^7 isomerase reaction in *Botrytis cinerea* (2), and several Oomycetes (3), but it apparently inhibits primarily the Δ^{14} reductase in *Ustilago maydis* (4). In higher plants which use the cycloartenol pathway in contrast to the lanosterol pathway of fungi, tridemorph inhibits the opening of the 9,19-cyclopropane ring characteristic of this pathway (5,6,7).

A study of the tridemorph inhibition of cultures of *Chlorella sorokiniana* was attractive for several reasons. *C. sorokiniana* is a member of Group IIIA of *Chlorella* based on sterol composition (8). This group is distinguished by production of ergosterol as the principal sterol (like most fungi) but unlike fungi, this group synthesizes ergosterol through the cycloartenol pathway of higher plants. *C. sorokiniana* is also unusual in that it synthesizes sterols with a methyl but not an ethyl at C-24. It is not known whether the lack of a second alkylation reaction is due to lack of the enzyme for the second alkylation or whether the product of the first alkylation could not serve as a precursor for the second alkylation. Triparanol and AY-9944 both produced an accumulation of 24-methylene compounds in other species of *Chlorella* (9,10), but neither inhibitor

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resulted in the accumulation of 24-methylene compounds in Chlorella sorokiniana (11,12), giving some reason to believe that C. sorokiniana could not produce the 24-methylene group. Tridemorph treatment of Chlorella would be expected to inhibit formation of the same end product (ergosterol) as in fungi. However, the cycloartenol pathway used by Chlorella will present a potential site of inhibition not present in fungi -- the opening of the 9,19-cyclopropane ring. This biosynthetic step usually occurs soon after the first alkylation reaction, meaning that inhibition of the opening of the cyclopropane ring should produce 24-methylene sterols if they are synthesized by Chlorella.

Methods

Algal cultures were grown on a standard glucose medium (8) in 1 ppm and 2.5 ppm of tridemorph (3.3 and 8.3 x 10^{-6} M) with rates of growth inhibition at 50% and 75%, respectively. Lipid was extracted from lyophilized cultures by extraction with chloroform/methanol (2:1) followed by saponification of the lipid and isolation of the sterol fraction from alumina column chromatography (8). The sterol fraction was analyzed by capillary gas chromatography on a 30 m column of SPB-1 (15) and by capillary gas chromatography-mass spectroscopy on a Finnigan-MAT model 4512 instrument as previously described (15).

Sterols of Chlorella sorokiniana cultured in presence of 1 ppm tridemorph.

Control cultures of C. sorokiniana contained ergosterol as the principal sterol as previously reported (8) with smaller amounts of ergost-7-enol and ergosta-5,7-dienol (Table I). A tridemorph concentration of 1 ppm completely inhibited synthesis of ergosterol with an accumulation of 24-methylpollinastanol and 24-methylenepollinastanol (Table I). The mass spectrum of 24-methylpollinastanol is presented in Table II and is identical to that previously reported (11). The mass spectrum of 24-methylenepollinastanol is also presented on Table II and is identical to that of the authentic compound (9). The data show that at 1 ppm of tridemorph, sterol biosynthesis is inhibited primarily at the opening of the cyclopropane ring since over 80% of the sterols from such cultures contain 9,19-cyclopropane rings. These data are in accord with work on carrot, tobacco, soybean (7) and bramble cell cultures (5) and with corn seedlings (6), all of which have sterol synthesis inhibited at the opening of the 9,19-cyclopropane ring. A third sterol detected in cultures inhibited by 1 ppm tridemorph was 5α-ergost-8(14)-enol, a sterol not previously reported from tridemorph-inhibited tissues. The capillary gc relative retention times of this sterol were similar to those of campesterol, 5α-ergost-8(9)-enol, 5α-ergost-8(14)-enol, 5α-ergosta-8(9),14-dienol, and 5α-ergosta-8(9), 24(28)-dienol. However, the mass spectrum clearly showed the presence of a monounsaturated C_{28} compound with few other distinguishing features, which is characteristic of 8(9) or 8(14) monoenes (13). An examination of the mass spectra (data not shown) of authentic 5α-ergost-8(14)-enol, 5α-ergost-8(9)-enol, 5α-stigmast-8(9)-enol, and 5α-stigmast-8(14)-enol showed that the spectra of the two C_{28} compounds and the two C_{29} compounds were practically identical except that the 8(9)-compounds had a base peak of m/z 43 while the 8(14)-compounds had a base peak of m/z 400 (or m/z 414). The compound from Chlorella had a base peak of m/z 400 suggesting its identity with 5α-ergost-8(14)-enol. Both packed column and capillary gas chromatography can distinguish between the two sterols, with the 8(14) compound having the shorter retention time on both polar and non-polar

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Table I. Gas Chromatographic Relative Retention Times and Quantitative Distribution of Sterols of *C. sorokiniana* Cultured with and without Tridemorph

		Sterol Distribution[a]		
	SPB-1	Tridemorph		
	RRT[b]	0	1 ppm	2.5 ppm
Ergosterol	1.21	85		
Ergosta-5,7-dienol	1.36	6		
Ergost-7-enol	1.38	9		
Ergost-8(14)-enol	1.27		18	
24-Methylpollinastanol	1.41		32	41
24-Methylenepollinastanol	1.38		50	35
24-Methylenecycloartanol	1.87			24

[a] as % of total sterol

[b] compared to cholesterol at 1.00

columns (14,15). The *Chlorella* compound had a RRT of 1.27 on SPB-1, identical with the RRT of 5α-ergost-8(14)-enol. The corresponding RRT of 5α-ergost-8(9)-enol was 1.33. The quantity of sample available did not permit NMR analysis. The data available indicate that one site of inhibition is at the opening of the 9,19-cyclopropane ring in agreement with previous work with plants using the cycloartenol pathway (5,6,7). This block is not complete at an inhibitor concentration of 1 ppm since some sterol is metabolized past the 9,19-ring opening step only to accumulate as 5α-ergost-8(14)-enol. It appears that little or no sterol is metabolized past this point with culture concentrations of tridemorph at 1 ppm.

Sterols of *Chlorella sorokiniana* cultured in the presence of 2.5 ppm tridemorph.

When cultures are grown in the presence of 2.5 pm of tridemorph, no desmethyl sterols were detected. The major sterols accumulating were 24-methylpollinastanol and 24-methylenepollinastanol which were accompanied by 24-methylenecycloartenol rather than 5α-ergost-8(14)-enol (Table I). 24-Methylenecycloartanol was identified by its characteristic mass spectrum and RRT (Table II). At 2.5 ppm of tridemorph, the opening of the cyclopropane ring appears to be completely blocked so that 5α-ergost-8(14)-enol cannot accumulate as it did at 1 ppm.

Table II. Mass Spectral Data[a] of some Sterols from Tridemorph Inhibited Chlorella sorokiniana

Fragmentation	Sterol[b] 1	2	3	4
$[M]^+$	400 (100)	414 (25)	412 (14)	440 (18)
$[M\text{-}CH_3]^+$	385 (28)	399 (65)	397 (20)	425 (26)
$[M\text{-}H_2O]^+$	382 (5)	396 (34)	394 (21)	422 (33)
$[M\text{-}33]^+$	367 (5)	381 (44)	379 (21)	407 (42)
$[M\text{-side chain}]^+$	273 (19)	287 (32)	287 (12)	315 (6)
$[M\text{-ring A + part of B}]^{+c}$	---	302 (18)	300 (11)	300 (28)
$[M\text{-side chain + water}]^+$	255 (9)	269 (31)	269 (15)	297 (11)
base peak	400	43	55	55

[a]reported as m/z (relative intensity)

[b]1 = ergost-8(14)-enol
2 = 24-methylpollinastanol
3 = 24-methylenepollinastanol
4 = 24-methylenecycloartanol

[c]due to cleavage of 9,19-cyclopropane ring and ring B

Tridemorph inhibits the opening of the 9,19-cyclopropane ring Chlorella sorokiniana as it has been reported to do in other plants synthesizing sterols by the cycloartenol pathway (5,6,7). It apparently inhibits the metabolism of 5α-ergost-8(14)-enol at a concentration of 1 ppm, an effect not previously reported. Although 5α-ergost-8(14)-enol can be easily distinguished from campesterol on mass spectroscopy, the two can be easily confused in gas chromatography. The frequent occurrence of campesterol in higher plants may have obscured the presence of ergost-8(14)-enol.

Significance of research results.

This work provides some additional insight into sterol synthesis of Chlorella sorokiniana, an organism differing from most plants and most other Chlorella species in that it apparently is unable to synthesize the C-24 ethyl group. This could be due to the lack of the enzyme necessary for the second alkylation reaction or it could be due to the lack of the necessary 24-methylene precursor for the second alkylation reaction. Previous work with other inhibitors with

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Chlorella sorokiniana have produced no accumulation of 24-methylene precursors as they have in other species of Chlorella leading to the speculation that Chlorella sorokiniana cannot synthesize the 24-methylene group. This work shows clearly that when inhibited with tridemorph, Chlorella accumulates 24-methylene compounds at both inhibitor concentrations tested. The current work suggests that the enzyme for the second alkylation is either missing or is ineffective in Chlorella sorokiniana.

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Chapter 18

Role of Sphingomyelin in Cellular Cholesterol Homeostasis

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When the ratio of sphingomyelin to cholesterol in cellular membranes is disturbed, cellular cholesterol metabolism is modulated. This report reviews some of the disturbances in cholesterol biosynthesis which occur after treatment of cells in culture with exogenous sphingomyelinase from *S. aureus*. Treatment of LDL with the same sphingomyelinase preparation results in >90% reduction of the sphingomyelin content of the LDL particle and greatly enhances processing of LDL.

There are many early reports in the literature which point out the special relationship that cellular levels of cholesterol and sphingomyelin (SM) bear to each other, these have been cited in two most excellent and recent comprehensive reviews on the structure, function, and cellular metabolism of sphingomyelin (*1*,*2*), and the reader is referred to them for information on this aspect. This report will focus on the effects on cellular cholesterol metabolism resulting from the disturbance of the ratio of cholesterol to sphingomyelin in membranes. Most of the cellular SM and cholesterol is located in the plasma membrane (*3*,*4*). Little is known of the regulatory factors which influence SM turnover and the effects on cholesterol metabolism, but some products of SM metabolism, e.g. sphingosine, are postulated to perform a signaling molecule function, and there has been much interest generated in studying the formation and disappearance of this substance (*5*). Generally, the higher level of SM in a membrane the greater the concentration of cholesterol in that membrane. Since each of these substances will greatly affect membrane structure, it is to be expected that the function of membrane embedded proteins will also be affected.

A most effective way to disturb the ratio of SM to cholesterol in the plasma membrane is to add a preparation of neutral sphingomyelinase (SMase)

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to the medium of cells in culture . This enzyme carries out the following reaction on SM in the plasma membrane.

sphingomyelin → ceramide + cholinephosphate.

The first work that reported on the effects of disturbance of the ratio of SM to cholesterol in the plasma membrane using exogenous SMase was that of Slotte and Bierman (*6*). They added neutral SMase from *Staphylococcus aureus* to the medium of fibroblast cells in culture, and observed in a short time, a rise in AcylCoA-cholesterol-acyl- transferase (ACAT) activity, which resulted in increased cellular cholesterol ester mass, and a reduction in endogenous cholesterol synthesis from acetate. They postulated that lowering of plasma membrane levels of SM resulted in a redistribution of cholesterol from the plasma membrane to the cell interior thereby accounting for the increase in cellular cholesterol ester mass and increased ACAT activity. The down-regulation of cholesterol synthesis was suggested to be the result of movement of cholesterol to a putative intracellular regulatory site. Subsequent work from Slotte and collaborators have reinforced these observations using the technique of cholesterol oxidase treatment (*7,8*). Exposure of cells to this enzyme allows only the cholesterol in the outer membrane to be oxidized while that in the cell interior is resistant to oxidation (*9*).

The observations with fibroblasts were also essentially duplicated in several cell lines, e.g. neuroblastoma cells (*10*) and baby hamster kidney cells (BHK-21) (*11*). In the latter case, a rapid resynthesis of SM was also observed within three to four hours, which was accompanied by a reversal of the changes elicited by SMase action. In fibroblasts (*12*) the effects of SMase treatment on the movement of plasma membrane cholesterol to the cell interior could not apparently be reversed by the addition of high density lipoprotein (HDL_3) into the medium. Slotte and collaborators examined the effect of the external application of SMase to fibroblasts from Nieman-Pick-C patients (*13*). The mutation in these cells appears to be localized to a defective intracellular transport of cholesterol, in that LDL derived cholesterol accumulates in the lysosomes and is unable to be esterified by ACAT, or to be involved in the down-regulation of endogenous cholesterol synthesis observed with normal fibroblasts (*14,15*). They found that NP-C fibroblasts responded to SMase treatment with movement of plasma membrane cholesterol to the cell interior with subsequent increases in ACAT activity and down-regulation of cholesterol biosynthesis from acetate. Thus, it appeared that although there was an obvious defect in cholesterol transport it did not involve the movement of cholesterol from the plasma membrane to the cell interior.

The above observations showing a link between SM and cholesterol metabolism have been supported by earlier reports which describe the effects of the addition of SM to fibroblasts in culture. It was observed that the addition of SM to fibroblasts caused effects opposite of those following SMase addition, i.e. cholesterol synthesis was stimulated with concomitant inhibition of the binding and uptake of low density lipoprotein (*16,17*). In addition HMGCoA reductase activity was stimulated (*16*). LDL uptake also inhibited endogenous SM synthesis (*18*). All of the foregoing reports emphasized the possibility that the close connection between levels of SM and cholesterol in the plasma membrane may play an important role in cellular phospholipid and cholesterol metabolism. They also suggested that disruption of this relationship

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could modulate normal mechanisms which maintain cellular cholesterol homeostasis.

Our interest in this problem arose from the effect of SMase activity on the down-regulation of cholesterol biosynthesis. We found that only acetate incorporation into cholesterol was inhibited, but not that of squalene or mevalonic acid thereby indicating that the site of inhibition was at the HMG-CoA reductase. We observed that addition of neutral SMase from *S. aureus* to the medium of a variety of cells in culture, e.g. rat intestinal epithelial cells (IEC-6), rat hepatoma cells (HEP G-2) and human skin fibroblasts (GM-43) resulted in significant inhibition of HMG-CoA reductase (HMGR) activity (*19*) (Figure 1). We could also reproduce all of the observations of Slotte and collaborators regarding the rise in ACAT activity and cholesterol ester levels after SMase treatment (*19*). The inhibition of reductase activity was dependent on the concentration of SMase and the duration of exposure. Addition of SMase to cell extracts had no effect on HMGR activity showing that intact cells were involved in the inhibition. Products of SMase action, e.g. ceramide or choline phosphate, did not appear to be responsible for the inhibition (*19*).

We considered the possibility that removal of cholesterol from the plasma membrane to the cell interior may have facilitated generation of regulatory polar sterols and that these might be involved in the decrease of reductase activity. HPLC and thin layer chromatographic analysis of the acetate labeled non-saponifiable lipid extract after SMase treatment, showed a significant increase in radioactivity in the polar sterol zone, in which several known inhibitors of reductase comigrate. A link between the increased level of polar sterol and decreased reductase activity was supported by our observation on the effect of ketoconazole, a known inhibitor of Cytochrome P-450 reactions and of regulatory oxysterol formation (*20-22*). As shown in Figure 2, pretreatment with 30μM ketoconazole completely prevented the inhibition of HMGR activity. Concomitantly we observed that ketoconazole inhibited the increase in oxysterol formation by SMase. Ketoconazole had no effect on the ability of SMase to hydrolyze SM. Other inhibitors of oxysterol formation or action, e.g. U1866A, progesterone also prevented the inhibitory effect of SMase treatment on HMGR activity (*19*).

We tested whether agents which facilitate the efflux of cellular cholesterol would reverse the effects of treatment by SMase from *S. aureus* on reductase activity. Treatment of cells with small unilamellar vesicles (SUV) of dioleyl phosphatidyl choline has been shown to enhance the efflux of cholesterol from cell membranes (*23*). We found that treatment of IEC-6 cells with SUV greatly accelerated cholesterol efflux into the medium and reversed the inhibitory effects of SMase treatment on reductase activity and the stimulation of ACAT activity (Table 1).

When HDL_3 the physiological acceptor of cholesterol efflux from cells was added to the medium, the inhibitory effect of SMase on reductase activity was also reversed (*19*). These effects with cholesterol acceptors supported the possibility that inhibition of reductase activity following SMase treatment was the result of movement of cholesterol from the plasma membrane to a regulatory site where conversion of cholesterol to a regulatory molecule could occur. Reversal of this movement to the cell interior could be overcome by the presence of cholesterol acceptor molecules in the medium. Our observations with ketoconazole suggest that the regulatory molecule may be an oxysterol. We were unable to rule out the possibility, however, that the cholesterol acceptor molecules in the medium may also have prevented the inhibition of reductase by removing the putative regulatory oxysterol. This would also explain the increased activity of HMGR when SUVs or HDL_3 are added to the medium.

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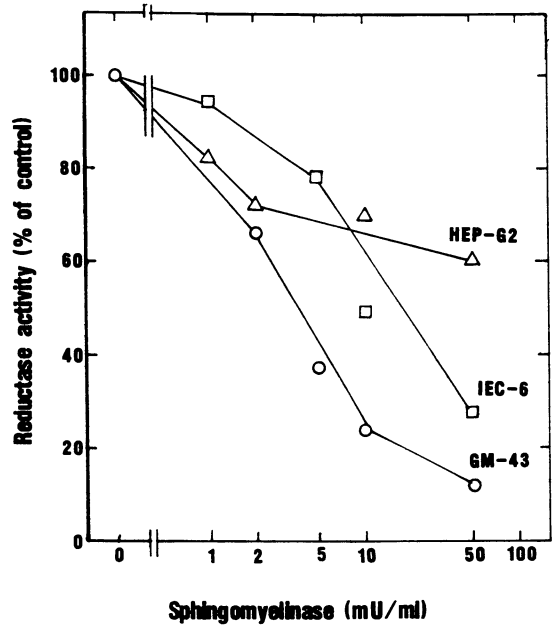

Figure 1. The effect of SMase on reductase activity in various cell lines. Experimental details and growth conditions as described in reference (*19*). (Reproduced with permission from ref. 19. Copyright 1991 Journal of Lipid Research.)

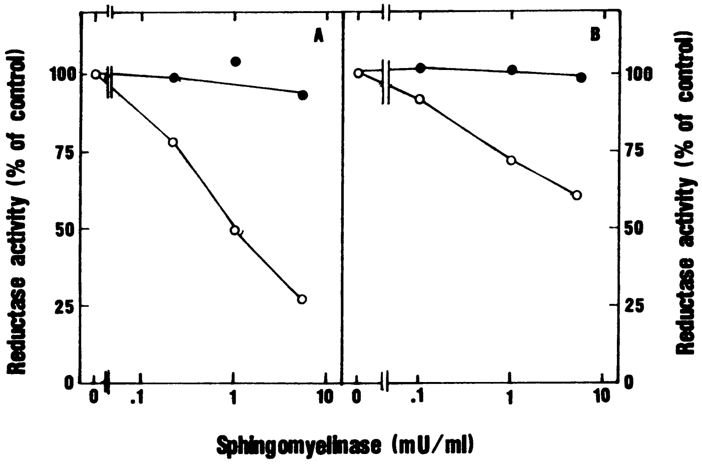

Figure 2. The effect of ketoconazole on the inhibition of reductase activity by SMase in IEC-6 cells. Experimental details and growth conditions as described in reference (*19*). (Reproduced with permission from ref. 19. Copyright 1991 Journal of Lipid Research.)

Table *I*

Effect of Small Unilamellar Vesicles on Sphingomyelinase Action on Cholesterol Movement, Sphingomyelin Hydrolysis, and Reductase Activity in IEC-6 Cells

Analysis	Treatment Conditions			
	-SUV		+SUV	
	None	SMase	None	SMase
Cholesterol esters (dpm × 10^2)	56	130	24	25
[^{3}H] Cholesterol in medium (dpm × 10^3)	30	33	122	120
Residual Sphingomyelin (dpm × 10^3)	16	3	13	4
HMG-CoA reductase (pmol/min per mg protein)	202	105	395	399

Experimental details and growth conditions as described in ref. (19). Reproduced with permission from reference (*19*), copyright *J. Lipid Research* 1991.

Since lysosomal SMase has been proposed as being involved in the degradation of membrane sphingolipids, we examined the possibility that we might influence the level of SM in the plasma membrane by inhibiting the activity of endogenous lysosomal SMase. Presumably this would result in increased levels of SM in the plasma membrane which would lead to enhanced cholesterol synthesis and HMGR activity. We tested the effect of adding to the medium of IEC-6 cells in culture some drugs which are known to inhibit lysosomal SMase activity, e.g., chlorpromazine, desipramine and W-7 (*24,26*). We found that these substances increased HMGR activity but they were unable to prevent the inhibitory effect of exogenous SMase on reductase activity (*19*). These effects are complicated by the pleiotropic actions of these agents in that they are also calmodulin inhibitors, in addition to being direct modulators of phospholipid metabolism (*27-29*). These results suggest that the cholesterol released from the plasma membrane as a result of exogenous SMase action may be transported to the putative regulatory site via a pathway that does not involve lysosomal interaction, since a similar drugs, e.g. imipramine (*30*), were shown to inhibit down-regulation of cholesterol synthesis and mobilization of LDL cholesterol from lysosomes. The studies of Slotte et al. (*13*) referred to above with NP-C cells also support this concept.

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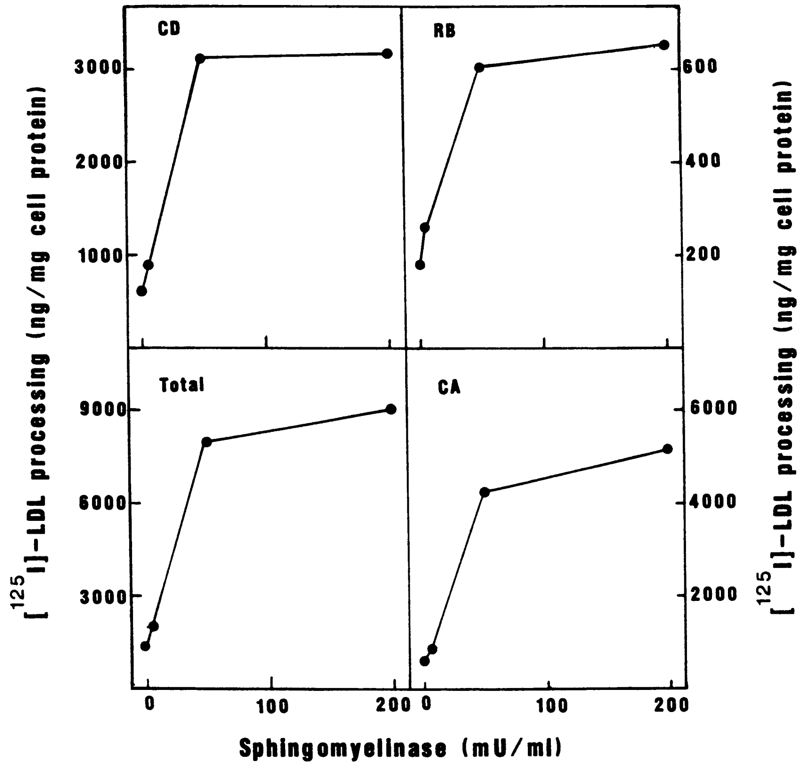

Figure 3. Effect of varying concentrations of SMase on the processing of ^{125}I-LDL by IEC-6 cells.

Since as noted above (*16*) the addition of SM to fibroblasts had the effect of inhibition of the binding and uptake of LDL, we examined the effect of exogenous neutral SMase on the binding, uptake and degradation of LDL in IEC-6 cells. IEC-6 cells were seeded (day 0) at 1 × 10^5 cells/well (35mm, 6 well plates) in 2 ml DMEM medium supplemented with 5% fetal calf serum (FCS Medium). The cells were refed 2 ml DMEM medium containing lipoprotein deficient serum (2mg protein/ml, LPDS medium) on day 3 and 4. On day 4, cells were fed LPDS medium with or without indicated concentrations of SMase. After 1h, ^{125}I-LDL (50 ug/ml) was added. Cells and medium were harvested for determination of cell degraded (CD), receptor bound (RB), cell associated (CA) radiolabeled LDL after 4h of incubation. Total processing is sum of CD, CA and RB. The data points presented are the mean of triplicate determinations. The results presented in Figure 3 indicate that addition of SMase from *S. aureus* to the culture medium caused a two to five fold increase in the total processing of LDL which included binding, internalization, and degradation. This increase in LDL processing was absent in cells devoid of LDL receptors. While this work was in progress an observation was reported at the 1991 FASEB meeting (*31*) which showed increased processing of LDL by fibroblasts after SMase treatment. In our further studies we observed that when ^{125}I-LDL was treated with SMase from *S. aureus* and purified via column chromatography the LDL particle had lost >90% of its sphingomyelin content.

This modified LDL displayed a >100% increase in binding affinity specifically to the LDL receptor at 4°. Thus the increased LDL processing appears to be due to an alteration of the LDL particle by *S. aureus* SMase which results in a particle with increased binding affinity to the LDL receptor (*32*). Further experimentation on the properties of this modified LDL particle is in progress.

The foregoing observations from our laboratory as well as those of others support the possibility that the ratio of cholesterol to SM in the plasma membrane plays an important role in cellular cholesterol homeostasis and that the level of SM in the LDL particle may also play a role in the overall cellular processing of LDL. Further examination of these concepts is actively being pursued in our laboratory.

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Chapter 19

Genetics and Molecular Biology of the Genes Functioning Late in the Sterol Biosynthetic Pathway of *Saccharomyces*

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The ergosterol biosynthetic pathway has been of interest due the fact that the pathway and the end product are the targets of the azole and polyene antifungals, respectively. More recently ergosterol has been implicated in some key regulatory processes of fungal cells. Genetic and physiological analyses of non-auxotrophic, ergosterol biosynthetic mutants have indicated the roles of ergosterol in providing an appropriate membrane structure and maintaining normal permeability characteristics. Efforts to ascertain the specific structural features necessary to provide the "sparking" or "hormonal" regulatory function have been investigated using gene disruption techniques. These procedures have shown that the C-24 sterol transmethylase (*ERG6*), the Δ^8-Δ^7 isomerase (*ERG2*), and the C-5(6) desaturase (*ERG3*) are not required for viability and, thus, are not required for the sparking function.

The yeast ergosterol biosynthetic pathway has been studied at the genetic and physiological levels for a number of years. With the advent of molecular biology, the ability to clone and analyze the genes and gene products has opened new areas of research. Investigators have explored this pathway for several reasons. The primary antifungals used today, the polyenes and azoles, bind to sterols in the plasma membrane or inhibit sterol biosynthesis, respectively. Recently, however, renewed interest in the pathway stems from the observation that a number of

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cellular proteins including Ras, are farnesylated (*1*). The farnesyl moiety is derived from a pre-squalene intermediate of the sterol biosynthetic pathway. Farnesylation presumably anchors the Ras protein into the plasma membrane and the absence of farnesylation results in this protein becoming cytosolic. In addition, the Gγ subunit is covalently bound to a novel isoprenoid, geranylgeranyl (2), a compound also derived from the pre-squalene part of the sterol biosynthetic pathway. Lastly, inhibitors of the ergosterol biosynthetic pathway might be useful as hypocholesterolemic drugs since many of the steps in animal and fungal sterol synthesis are the same. Little enzymology has been done in yeast as none of the biosynthetic enzymes late in the pathway have been purified. However, Gaylor's group at Dupont have published some very impressive work on enzymes late in the cholesterol biosynthetic pathway (*3-6*).

Our interest in the ergosterol biosynthetic pathway has been more narrowly focused and involves the conversions of lanosterol to ergosterol (Figure 1). Observations made over many years have indicated several alternate routes to the formation of the end product are possible. Rather than present all of these possibilities we have chosen to present only the primary biosynthetic route of S. cerevisiae. We are therefore interested in the physiology of the cell in which the end-product sterol, ergosterol, is replaced by precursor sterol. Lynn Miller made the key observation that nystatin resistant mutants of yeast had altered sterol content and this was followed by the isolation of sterol mutants in several laboratories (*7,8*). One of the remarkable properties of these mutants (*erg*2, *erg*3, *erg*5, *erg*6 - see Table I) was that they did not require exogenous sterols nor could they take up sterols from the growth medium when grown aerobically. A second set of nystatin resistant mutants complicating the early studies were the *hem* or *ole* strains which not only could not synthesize sterols but also could not desaturate fatty acids, synthesize methionine or respire. This set turned out to be heme auxotrophs (*9,10*).

Sterols are essential components of eukaryotic membranes influencing membrane fluidity, membrane permeability, the activity of membrane bound enzymes, and cellular growth rates (*11-15*). However, several reports have indicated additional roles for sterols. Bloch's group described the sparing effect of sterols where pairs of sterols have a synergistic effect on cell growth (*16*) and Parks' group described the sparking effect

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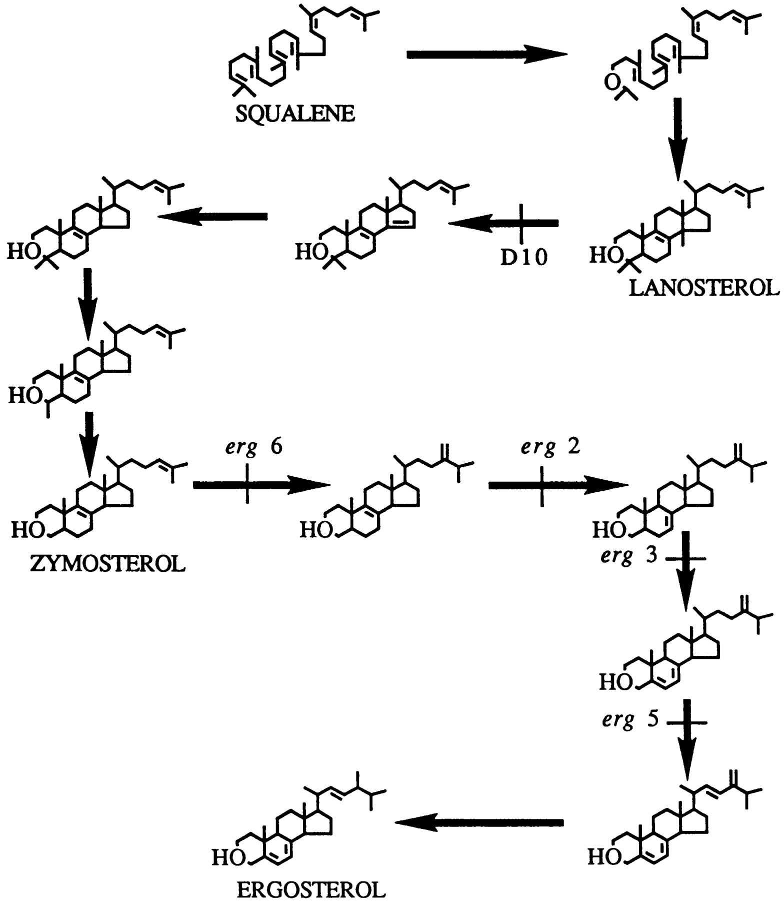
SQUALENE
D10
LANOSTEROL
erg 6
erg 2
ZYMOSTEROL
erg 3
erg 5
ERGOSTEROL
HO

Figure 1. The Sterol Biosynthetic Pathway from Squalene to Ergosterol in Saccharomyces cerevisiae. (Adapted from Parks, L.W. *CRC Crit. Rev. Microbiol.* **1978**, *6*, 301-341.)

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whereby "hormonal" or "sparking" levels of a particular sterol are required for growth (*17,18*). The sterol responsible for the sparking function may be the end product ergosterol or an ergosterol precursor. The sterol levels required to satisfy the sparking function (25nM) are insufficient to meet the cell membranes sterol requirements (the bulk membrane sterol). The bulk membrane sterol could be met by a number of ergosterol precursors, cholesterol, or even cholestanol (a sterol devoid of unsaturation).

The role of the sparking sterol was made clearer when Dahl et al. (*19*) demonstrated arrest of sterol-depleted cells of an ergosterol auxotroph in an unbudded G1 state. Cell budding and proliferation were reinitiated upon addition of limiting amounts of ergosterol or non-limiting levels of cholesterol. The data suggest that addition of hormonal levels of ergosterol (1nM) stimulate a protein kinase antigenically related to $pp60^{v\text{-}src}$ which enables the cell to exit G1. Quesney-Huneeus et al. (*20,21*) showed that cholesterol feeding of mammalian BHK-21 cells whose growth was suppressed by compactin inhibition of HMG-CoA reductase, restored the normal S-phase burst of DNA synthesis. This effect of cholesterol was independent of the cell's requirement for mevalonate (a precursor of cholesterol). Their data suggest that cholesterol may function as a sparking sterol in mammalian cells.

A controversy has arisen as to the specific structural requirements of the sparking sterol. Using a yeast strain containing two early lesions in the ergosterol biosynthetic pathway (such that no sterol molecules are made), Parks and colleagues (*17,18,22*) demonstrated that only sterols possessing a C-5(6) double bond or those capable of being desaturated at C-5 fulfilled the high specificity sparking requirement. Their studies demonstrated that S. cerevisiae auxotrophs can utilize cholestanol (5ug/ml) to satisfy their sterol growth requirement when small amount of ergosterol were present (10 ng/ml). Nes and colleagues (*23,24*) have disputed these findings an suggest that there is an absolute requirement for a sterol containing a C-24 methyl group in the sterol side chain. Their protocol used anaerobic cells which require sterols for growth and a metabolic inhibitor, 2,3-iminosqualene, which inhibits squalene cyclase and, therefore, lanosterol formation. McCammon et al. (*25*) have disputed Nes' findings . Their studies using *erg6* (deficient in C-24 methylation) demonstrated that virtually no [C^{14}-methyl] methionine incorporation into non-saponifiable lipids could be detected and concluded that C-24 methyl sterols are not essential to the sparking function. Recently, Nes et al. (*26*) and Haughan et al. (*27*) have observed an essential requirement for C-24 methyl and ethyl sterols in the fungus, Gibberilla and in the plant Apium, respectively.

Since the sparking sterol is required in such small amounts, studies using auxotrophic mutants and sterol synthesis inhibitors in feeding experiments have the possibility of having minute amounts of ergosterol or other sparking intermediate produced because of low level leakiness or incomplete inhibition. In addition, the complexity of sterol interconversions may yield some sparking sterol intermediate derived from an exogenous sterol. Some of these possibilities can be eliminated in strains where the genes specifying the sterol biosynthetic enzymes can be converted to null alleles by employing gene disruption techniques.

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Genetics of Sterol Mutants

The initial attempts to isolate mutants of the sterol biosynthetic pathway were conducted by screening for sterol requiring phenotypes. This approach was largely unsuccessful resulting in the isolation of *hem* or *ole* mutants primarily (*9,10*). Greater success was achieved by screening for nystatin resistant mutants. This approach is based on the fact that nystatin's antifungal properties result from its binding to membrane ergosterol and inducing lethal cell damage. A mutant which does not synthesize ergosterol and thus must substitute an ergosterol precursor into its membrane would be expected to be less affected by nystatin. Several early studies using this screening procedure produced a number of nystatin resistant mutants which were found to contain abnormal sterol profiles (*7,8,28*). Originally, such mutants were classified as *nyr* or *pol* mutants. In order to avoid confusion with DNA polymerase mutants, designation of these mutants has been changed to *erg* mutants. We will adhere to this terminology.

Four different sterol pathway mutations were isolated and characterized during these early studies. A fifth pathway mutant, *erg*4, which was unable to reduce the double bond at C-24(28) and accumulated ergosta-5,7,22,24(28)-tetraen-3β-ol, was also isolated but was found to be leaky. Since this mutant produced significant levels of ergosterol it was not pursued in further studies. Table I lists the mutants, the biosynthetic step

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blocked, and the sterol accumulation products for each of the *erg* strains. *erg*6 is unable to methylate the C-24 position of zymosterol resulting in the accumulation of C27 sterols. The *erg*2 mutation results in the formation of Δ^8 sterols indicative of a block in the Δ^8-Δ^7 isomerase while *erg*3 is unable to desaturate the C-5(6) position. Both the *erg*2 and *erg*3 phenotypes are easily recognized by the absence of the typical B ring diene system at C-5 and C-7 seen in UV spectra of ergosterol-producing strains. The *erg* 5 mutation results in the inability to desaturate the sterol side chain at 22(23). It has been shown that the level of nystatin resistance for these mutants is greater the earlier (Figure 1) the mutation in the pathway (*7*,*8*).

Table I. *erg* mutants of Saccharomyces cerevisiae with blocked biosynthetic steps and sterol accumulation products

Mutant	Blocked Step	Accumulated Sterols
*erg*2	Δ^8-Δ^7 isomerization	ergosta-8-en-3ß-ol ergosta-8,22-dien-3ß-ol ergosta-5,8,22 trien-3ß-ol
*erg*3	5(6) desaturation	ergosta-7,22-dien-3ß-ol ergosta-8,22-dien-3ß-ol ergosta-7,22,24(28)-trien-3ß-ol ergosta-8,22,24(28)-trien-3ß-ol
*erg*5	22(23) desaturation	ergosta-5,7-dien-3ß-ol zymosterol ergosta-7,24(28)-dien-3ß-ol
*erg*6	C-24 transmethylation	zymosterol cholesta-5,7,22,24-tetraen-3ß-ol

Genetic analysis of all four of these mutants has been performed (*7*). All four mutations are recessive in diploids constructed by mating to the wild type. Sporulation of the diploids resulted in nystatin resistance and altered sterol pattern segregating together in a 1:1 ratio with the wild type phenotype. Crosses between various combination indicated that all four genes were unlinked.

Permeability Studies of the *erg* Mutants

There is an extensive literature describing the role of sterols in biological membranes. They have been shown to influence critical membrane function such as permeability and the activity of membrane-bound enzymes (*29-31*). Our early interests regarding sterol pathway mutants concerned the physiological and biophysical effects of sterol substitution that could be measured functionally or structurally. Several of the mutants, (*erg*2, *erg*3 and *erg*6), in addition to a double mutant, *erg*6/2, which accumulates zymosterol, were investigated for altered permeability characteristics using a crystal violet dye uptake assay and sensitivity to mono-, di-, and trivalent cations (*13*). All strains tested showed increased dye uptake over a range of temperatures when compared to the wild type strain. The *erg*6/2 strain showed the greatest dye uptake indicating that zymosterol, the principle membrane sterol, is the least efficient sterol for maintaining normal permeability characteristics. Similarly, the studies exposing the sterol mutants to pulses of high concentrations of various cations indicated that the mutants suffered significantly greater losses of viability than did the wild type. Among the mutants *erg*3 was the least affected while *erg*6 and the *erg*6/2 double mutant were the most affected again indicating that later sterol intermediates are better at providing normal membrane permeability characteristics then early intermediates.

A separate study (*32*) of altered permeability characteristics of sterol mutants employed the double mutant, *erg*6/2, in experiments utilizing a unique application of electron spin resonance (ESR). In this study a water soluble nitroxide probe, 2,2,5,5 tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid (PCA), was introduced into yeast cells. This molecule freely enters all cells and is routinely used to determine cytoplasmic microviscosity. PCA labelled cells were then suspended in a $NiCl_2$ solution which eliminated label signals from extracellular aqueous regions by magnetic dipolar broadening. Thus, cytoplasmic PCA was the only remaining source of signal. Permeability was monitored by measuring the Ni^{2+} entry which is reflected by its magnetic dipolar line broadening effect on the internal PCA signal. The *erg*6/2 was shown to be significantly more permeable to Ni^{2+} than the wild type strain. These differences were noted in all growth phases of the cells.

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Membrane Structure Alterations of Sterol Mutants

ESR techniques were also employed in determining the effects on sterol substitution on membrane fluidity (*33*). In these studies a modified stearic acid molecule containing a nitoxyl group at the number 5 carbon position was inserted into the cytoplasmic membrane of wild type and mutant strains *erg*2, *erg*6 and *erg*6/2. Analysis of the ESR spectra produced by the nitroxyl probe yields information regarding the motional freedom of the fatty acid chain. This motional freedom is calculated as an order parameter, S, where lower values indicate higher motional freedom and higher values reflect more restrictive motion indicative of a more ordered or rigid membrane. Sterols have been shown to effect membrane order parameter in several systems and it was of interest to apply this analysis to our ergosterol mutants. Results indicated that *erg*6 and *erg*2 had significantly higher order parameters compared to the wild type strain while the double mutant, *erg*6/2, was significantly more rigid than either of the two single mutants. This relationship was shown in both exponential and stationary phase cultures. These results correlate with the permeability studies which indicate that the earlier the block in the ergosterol pathway the less effective the sterol intermediates are at providing normal membrane function and structure.

It is of interest to note a similar but more extensive ESR study of membrane fluidity (*34*) has been conducted using a cytochrome P450-deficient mutant strain (D10) of C. albicans (*35,36*). The lesion in this strain results in the inability to remove the C-14 methyl group from lanosterol (Figure 1). Thus, this mutation results in a strain where the block in the ergosterol pathway occurs earlier than the blocks in the *erg* mutants discussed thus far. Mutants of this step (*erg*11) have not been isolated in S. cerevisiae unless they are leaky (*37,38*), are accompanied by a second mutation in the C-5(6) desaturase (*37,39*), or require exogenous ergosterol (*40*). Membrane order parameter analysis of strain D10 using the 5DS probe indicated a more rigid membrane than found in *erg*6 or *erg*2. This increased rigidity was found to exist to a greater extent deeper in the membrane when stearic acid probes labeled at the 7 or 10 carbon positions were employed. Microviscosity measurements of the membrane at the 16 carbon position also indicated a significantly more rigid membrane in strain D10 when compared to the wild type. These results further corroborate and extend observations made with the *erg* mutants of S. cerevisiae regarding earlier sterol intermediates being increasingly unable to provide an appropriate membrane structural environment.

Physiological Characteristics of the *erg* Mutants

A number of investigations have described the effects of sterol content on cellular physiological properties such as growth characteristics (*15,36,41,42*). In one report (*41*), a sterol mutant of S. cerevisiae allelic to *erg*3 showed a significantly decreased growth rate on ethanol and somewhat diminished ability for fermentative growth. Subsequent to these earlier reports a thorough analysis of the growth characteristics of several sterol mutants of S. cerevisiae has yielded some interesting results (*15*). Using both mean generation time and growth yield analysis, *erg*2 and *erg*3 were shown to have characteristics similar to the wild type while *erg*6 and *erg*6/2 showed significant decreases in growth on glucose. When ethanol or glycerol was substituted for glucose, *erg*6 and *erg*6/2 show virtually no growth indicating a loss of respiratory capacity. These effects on ethanol growth are due primarily to the inability to utilize ethanol as an energy source rather than a direct membrane effect since cells grown on glucose plus ethanol resulted in only slightly reduced growth. Again, using growth capabilities as a parameter, the lack of a C-24 transmethylase function has a more deleterious effect than either the Δ^8- Δ^7 is isomerase or the C-5(6) desaturase.

Further studies employing a strain carrying a disrupted *ERG*6 gene have indicated several additional physiological consequences resulting from the inability to methylate zymosterol (*42*). In addition to the anticipated resistance to nystatin this strain was shown to possess a hypersensitivity to cycloheximide, and a three-fold lower mating frequency in *erg*6/*erg*6 matings when compared to *ERG*6/*ERG*6 matings. Tryptophan uptake was also shown to be affected by the functional removal of the C-24 transmethylase gene. At 22°C, tryptophan uptake in the disrupted strain was reduced six-fold compared to the wild type. This decrease in uptake resulted in a loss of viability of tryptophan auxotrophs of the disrupted strain. At 37°C, tryptophan uptake was reduced by 2.5-fold which permitted sufficient uptake to allow the survival of a trp$^-$ disrupted strain. Similar, though less severe, deficiencies in tryptophan uptake have been observed in *ERG*2 and *ERG*3

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disruptions (Bard, M., Indiana University-Purdue University at Indianapolis, unpublished data).

Cloning of the *ERG*2, *ERG*3, and *ERG*6 Genes

Initial attempts to transform the *erg* mutants by complementation were not successful due to the pleiotropic membrane defects of these strains. Very low frequencies of genetic transformation, less than one transformant per ug of plasmid DNA were obtained. To increase the frequency of transformation, the procedure of Ito et al. *(43)* was modified by Gaber et al. (*42*) by harvesting the cells very early in log phase (Klett of 35 or less). This modification reduced the possibility of the cells acquiring characteristics that inhibit transformation. Other modifications included decreasing the polyethylene glycol concentration form 40% to 36% and increasing the amounts of plasmid DNA and carrier DNA twenty-fold and three-fold, respectively.

Each of the three late ergosterol biosynthetic genes were isolated from a library of wild type yeast DNA fragments constructed into the shuttle vector, YCp50. Selection was based on the ability to suppress cycloheximide sensitivity in the recipient strains. Almost all cycloheximide resistant transformants were nystatin sensitive and synthesized ergosterol. Attempts to integrate the cloned DNA fragments at their respective wild type loci utilizing the integrating vector, YIp5, confirmed that the cloned DNA encoded the *ERG* genes and not non-allelic suppressors. A segregation pattern of 2:2 was observed for each of the three genes integrated indicting genetic linkage between the integrated plasmid containing the ergosterol gene and the specific gene locus on the yeast chromosome. Once the genes were cloned, gene disruption techniques were used to generate null alleles and cells possessing these null alleles were observed for viability.

Five clones of the *ERG*6 gene were obtained and the gene was localized on a 3.1 kb *Kpn*I-*Pvu*II DNA fragment. Gene disruption entailed the removal of a 400 bp *Sal*I-*Xba*I DNA fragment (Figure 2) into which a 1.9 kb DNA fragment containing the *LEU*2 gene was inserted. Disruption of the genomic *ERG*6 gene was accomplished by the one-step gene disruption protocol of Rothstein *(44)*. A second, larger deletion that removed at least 90% of the *ERG*6 coding region was also constructed and in both cases the *erg*6Δ strain was viable. Southern hybridizations

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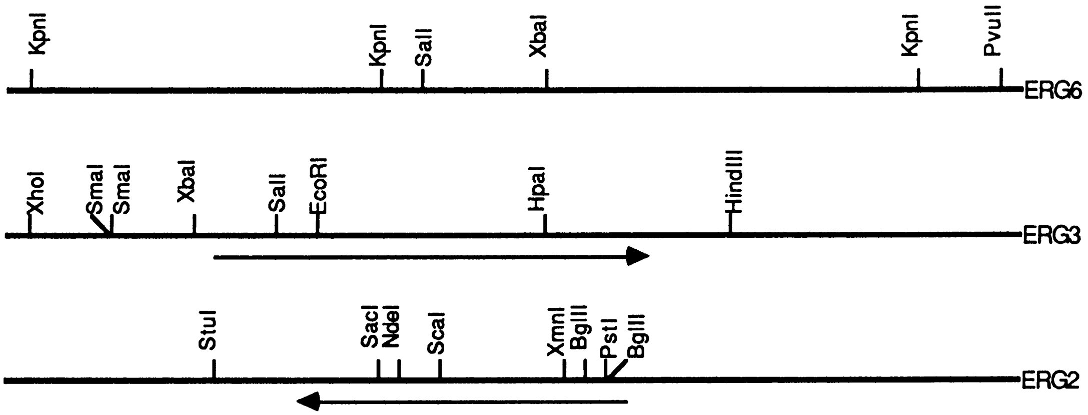
KpnI
KpnI
SalI
XbaI
KpnI
PvuII
ERG6
XhoI
SmaI
SmaI
XbaI
SalI
EcoRI
HpaI
HindIII
ERG3
StuI
SacI
NdeI
ScaI
XmnI
BglII
PstI
BglII
ERG2

Figure 2. Restriction Endonuclease Sites of the Yeast ERG6, ERG3, and ERG2 Genes. Arrows indicate the location of the sequenced open reading frames.

confirmed both disruptions. Only when the *erg*6 deletion was created in a strain which also contained the *trp*1 lesion was the resulting strain inviable. The *erg*6 *trp*1 double mutant was found to be cold-sensitive for tryptophan uptake allowing growth at 37°C but not at 30°C or 22°C.

Only one clone of *ERG*3 was obtained by transformation (*45*). The gene was subcloned into a 2.5 kb *Bam*HI-*Hin*dIII fragment. The coding sequence begins approximately at the *Xba*I site and ends at the indicated arrow (Figure 2). DNA sequence analysis of the *ERG*3 gene indicates a 365 amino acid polypeptide with a hydrophobic 24 amino acid sequence which may be involved in anchoring the polypeptide into the ER. Gene disruption involved removing a 0.8 kb *Sal*I-*Xho*I DNA fragment and in its place ligating the 2.0 kb DNA fragment containing the *LEU*2 gene. This disruption removed the entire promoter and the first 75 amino acids of the coding sequence. The transformed strain containing the deletion-substitution was viable suggesting that *ERG*3 is also non-essential for yeast viability.

The observation that morpholines inhibit the C-8 sterol isomerase suggested that *ERG*2 may be an essential gene (*46*). Using the same transformation protocol, four clones of the *ERG*2 gene were isolated and the gene was localized on a 1 kb *Bgl*II-*Stu*I DNA fragment shown in Figure 2 (*47*). DNA sequence analysis indicated three possible reading frames of 222, 206, and 193 amino acids. The *ERG*2 gene was disrupted by again inserting the *LEU*2 gene into the coding sequence at the *Nde*I site. This disruption bifurcates *ERG*2 approximately 60% into the coding sequence. As before, the disrupted gene did not give rise to an inviable strain.

Our results indicating that the three *ERG* genes are not essential for viability is not surprising given the ease with which mutants of these genes have been obtained. Our analyses indicate that it is not necessary to postulate that mutant strains of *erg*2, 3, and 6 are necessarily leaky.

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